IRRIGATION, NUTRITION, AND EVALUATION OF ADVANCED BREEDING LINES TO IMPROVE FREEZE HARDINESS OF CITRUS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1997

This dissertation is dedicated to Thea Margaret Edwards who, in short, stopped me from walking away from it all.

ACKNOWLEDGMENTS

I express my sincere appreciation and thanks to Dr. Fred Davies, committee chairman, for his supervision, advice, and patience during the completion of this dissertation. I especially thank him for his superior editing skills and for allowing me to pursue research on citrus freeze hardiness that didn't always overlap with his main interests. In addition, I appreciated his confidence in allowing me to lecture for both his *Introduction to Citrus Culture* and *Citrus Production Management* courses many times.

I also express my sincere appreciation and thanks to Dr. Wayne Sherman, committee cochairman, for his insight into citrus breeding and for providing funding which helped to support many of my research endeavors. I am also indebted to Dr. John Davis, Dr. Gloria Moore, and Dr. Jeff Williamson for participating on my graduate committee and research support. In particular, Dr. John Davis provided lab space and technical expertise for much of the molecular biology work found in this dissertation and Dr. Gloria Moore was always ready to provide critical advice and was very generous with laboratory supplies and equipment. In addition, I thank Dr. Rebecca Darnell for the generous use of her lab and growth chamber space which was critical to many experiments.

I also owe a great debt to Michael Maurer and Laura Guazzelli who both helped initiate me to the University of Florida and give me the 'inside scoop' that

every graduate student needs. I am certain they saved me six months of wandering about aimlessly. Dr. Haiguo Wu and Tess Korhnak also helped me considerably at the lab bench with technical advice and training.

I am also very fortunate to have made so many friends here that I could not possibly list them all. These individuals have made my personal life, which has been trying of late, bearable which in turn allowed me to focus on science. Special thanks along these lines go to Deb McGrath, Sandra McDonald, John Coe, Shahab Hanifkahn, Tina Knutsen, John Westine, Mary Mason, Courtney Weber, Betsy Bihn, John Davis, Kathy Davis, Diane Luth, Brent Cooley, Jennifer Wolfe-Cooley, John Melton, Jon Johnson, Barbara Crane, Dave Nolletti, Chris O'Brien, and Carla Lyerly.

Finally, I offer my sincere thanks and gratitude to my parents for their continuing support of my educational pursuits. They have been the one constant in my life.

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August 1997

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A factorial experiment with three irrigation schedules based on the presence or absence of growth flushes and three nitrogen application frequencies was conducted in 1994 and 1995. Irrigation scheduling had a significant effect on freeze acclimation of young 'Hamlin' orange trees (*C. sinensis* (L.) Osb.), during periods without significant rainfall. Irrigating when soil water depletion (SWD) exceeded 20% through the 1st growth flush and > 45% SWD for subsequent flushes resulted in significant increases in freeze hardiness as determined by the leaf disc electrolyte leakage (EL) method compared to trees that received more water. Trunk diameter and tree height were generally similar regardless of N application frequency or irrigation treatment, but irrigation had a significant effect on the occurrence of growth flushes during 1994.

Advanced USDA intergeneric hybrid scion breeding lines 'US 119' and 17-11 were compared to 'Hamlin' orange and satsuma mandarin for differences in freeze hardiness during 4 winters from 1993-1997. Both 'US 119' and selection 17-11 were hardier than 'Hamlin' orange. Both showed similar freezing tolerance to satsuma mandarin. Selection 17-11 was significantly hardier than satsuma or 'US 119' during several dates tested during the 4-year study. Trunk diameter and plant height were similar for 'US 119' and 17-11.

In order to look at very early changes during freeze acclimation, *P. trifoliata* seedlings were examined at 0, 6, 24, 168, and 504 h for changes in EL and xylem water potential following exposure to 10 °C. Decreases in EL occurred within 24 h in the leaves and continued to decrease significantly up to 168 h. Xylem water potential initially decreased from –0.6 to –2.0 MPa after 6 h then returned to –0.6 MPa after 168 h. *P. trifoliata* seedlings also acclimated more rapidly than either similarly treated *C. grandis* or *C. jambhiri* seedlings based on periodic freeze tests. Accumulation of anthocyanins was also correlated with exposure to 10 °C and long days.

These results suggest that modified irrigation practices and integration of hardiness traits from advanced breeding selections could improve levels of freeze protection in the field. In addition, *P. trifoliata* seedlings responded to acclimating temperatures much earlier than previously thought.

CHAPTER I
INTRODUCTION

Plants are limited world wide primarily by low temperature (Parker, 1963). Citrus, characterized as a subtropical evergreen (Swingle and Reese, 1967), is limited to production between 40° N and S latitude based primarily on temperature (Yelenosky, 1985). Within this area commercial citrus production is subject to periodic severe and economically devastating freezes.

Historically, some protection has been provided through rootstock and scion selection, but the majority of protection has been in the form of one-time measures taken just prior to and during an actual freeze. Petroleum heaters (Parsons et al., 1982), wind machines (Turrell, 1973), windbreaks (Martsolf et al., 1986), and soil banking (Jackson et al., 1983) have all been used with some success for freeze protection of citrus in the past. Currently, the most commonly used and economical freeze protection of citrus is microsprinkler irrigation (Ferguson and Taylor, 1993). Moreover, microsprinkler irrigation in conjunction with tree wraps provides particularly good freeze protection for young citrus trees (Rieger et al., 1985).

However, these methods of freeze protection have their drawbacks. Soil banking and petroleum heaters have high labor costs and soil banks have the potential to damage trees. The likelihood of brown outs during a freeze requires

an irrigation system with an alternate power source. In addition, even with the best execution of these protection methods, billions of dollars have been lost during the last century and the practical northern limit of the citrus industry has moved south.

It has been suggested for a number of years that curtailing fertilization (Hume, 1956; Rasmussen and Smith, 1961) and or irrigation (Husain, 1958; Yelenosky, 1979) in the fall can increase citrus freeze hardiness by promoting quiescence. However, results of research on nutrition and irrigation scheduling practices conflict. This is often due to one-time post freeze evaluation of damaged citrus, which is impossible to replicate in the field, although it can yield useful information about a given freeze. In addition, citrus species, canopy size, tree age, and cultural practices affect freeze hardiness. Therefore, a better understanding of how irrigation scheduling and fertilizer application frequency affect freeze hardiness is needed.

Another method for improving citrus freeze hardiness in general is the introduction of improved hardiness and acclimation traits through breeding. The USDA in Orlando, FL has had an ongoing citrus breeding program for decades (Barrett, 1981; 1985; 1990a; 1990b; 1994; as reviewed by Swingle and Reece, 1967). Barrett has produced many advanced selections containing desirable traits for the citrus industry. Two hybrids, 'US 119' and selection 17-11, have the potential of being extremely freeze hardy. These hybrids need to be examined for not only maximal freeze hardiness, but for acclimation and deacclimation rates that can be critical for citrus survival during a freeze.

Finally, although much research has been done on citrus acclimation, few studies have looked at changes during the first few hours when citrus is exposed to low acclimating temperatures. *P. trifoliata*, a very hardy deciduous relative of citrus, is a good candidate for understanding what early physiological changes might be important in freeze acclimation.

This research will have application to several facets of the citrus industry. Data presented on the effects of irrigation scheduling and nutritional cultural practices on freeze hardiness could change cultural recommendations made for growing young citrus trees in Florida. Evaluations of 'US 119' and 17-11 not only confirm their freeze hardiness potential, but suggest that freeze acclimation rates may vary among these selections. Findings on early physiological and molecular events during acclimation of *P. trifoliata* could also expand our knowledge of basic freeze hardiness and acclimation processes.

CHAPTER II
REVIEW OF LITERATURE

Introduction

The major limiting factor in plant distribution worldwide, water availability aside, is temperature (Lyons et al., 1979; Parker, 1963; Sakai and Larcher, 1987). In addition, geographic distribution of plants is most often limited by low temperatures (Parker, 1963).

Plants are either acclimating or non-acclimating with respect to freeze tolerance. Alberdi and Corcuera (1991) described freeze acclimation as "a response to cold which minimizes damage and improves the fitness of the plant" (p. 3178). These responses occur during acclimation by the induction of either tolerance or avoidance mechanisms (Levitt, 1980). Intracellular freezing is always lethal due to irreversible membrane damage and disruption of cellular contents; however, extracellular freezing is tolerated in some plant species (Burke et al., 1976; Levitt, 1980). Acclimated plants exposed to freezing temperatures may also avoid intracellular freezing by the exclusion of nucleators (supercooling) (George and Burke, 1984), or by tolerating the extreme dehydration caused by the removal of all free water from the cytosol (non-

supercooling) (Levitt, 1980). Other physical changes that have been correlated with freeze acclimation in plants include a shift from saturated to unsaturated membrane lipid species (Miquel et al; 1993; Palta and Weiss, 1993; Steponkus, 1990; Uemura and Yoshida, 1984), increases in starch, sugar, and proline concentrations (as reviewed by Guy, 1990), increased production of cell wall proteins such as extensin (Weiser et al., 1990), and increases in plant secondary metabolites such as anthocyanins (Camm et al., 1993; Leyva et al., 1995; Singh et al., 1995; Steponkus and Lanphear, 1969; Christie et al., 1994). Although these and many other changes have been characterized individually, integration of this knowledge into a cohesive theory has yet to be accomplished (Alberdi and Corcuera, 1991; Guy, 1990; Steponkus, 1984).

Freeze acclimation occurs naturally in many temperate-zone plants by gradual exposure to low temperatures and perception of a decreased photoperiod. However, specific night length (actually a more correct term than short days since P_{fr} is degraded during darkness) requirements vary greatly with species. The relationship between the onset of true dormancy and the process of freeze acclimation is unclear. Certainly, a completely dormant temperate woody plant such as *Salix* sp. is very hardy in the middle of winter (Becwar and Burke 1982), but less is known about the induction of freeze hardiness. *Populus deltoidies* will set a dormant apical bud under an 8 h short day (SD) photoperiod, but leaves on the plant remain intact and green until exposure to low temperatures (John Davis, Gary Coleman, personal communication), suggesting signaling roles for both photoperiod and temperature in freeze acclimation.

Plants adapted to subtropical climates typically do not respond to changes in photoperiod as an impetus for freeze acclimation, but instead acclimate and deacclimate solely on tissue temperatures. Citrus, a subtropical evergreen, possesses many of the same general freezing tolerance and avoidance mechanisms as temperate woody perennials, but to a much lesser degree. Many temperate woody species, when fully freeze acclimated, can survive immersion in liquid nitrogen with no damage (Becwar and Burke, 1982). In contrast, the maximum leaf freeze hardiness for sweet orange is only about –6.7° C (Yelenosky, 1990). Much less is understood about hardiness mechanisms and timing in subtropical citrus as compared to many temperate woody perennials. Unfortunately, even after extensive research, no model of citrus freeze acclimation is comprehensive enough to be a predictive tool in the field (Yelenosky, 1985).

Economic Importance of Freezing on the Citrus Industry

Commercial citrus production occurs within 40° north and south of the equator, where infrequent freezes and availability of suitable land allow (Swingle and Reece, 1967). Many citrus production areas have been repeatedly subjected to severe freezes including Japan, Spain, Israel, Greece, northern Mexico, and the United States (Yelenosky, 1985). Most areas of United States production are subject to severe freezes with great economic consequences (Yelenosky, 1985).

In Florida, where there are currently 347,101 ha of citrus and 107 million trees (Commercial Citrus Inventory, 1996), freeze losses have the potential to be devastating and have been severe in the past (Table 2.1). Since 1766, 23 severe freezes have damaged commercial citrus crops to varying degrees (Rogers and Rohli, 1991). Freezes in 1894 and 1895 initiated a general migration southward of the citrus industry. With subsequent periodic freezes this migration has continued southward, especially following multiple severe advective freezes in the 1980s (Fig. 2.1). The fact that only 29.5% of the 342,081 ha of citrus reported in the 1980 citrus census is still in production in 1996 emphasizes the continuing extent of this southern shift. Freeze damage in the 1980s was the single greatest contributing factor in causing Florida production to fall behind that of Brazil as world leading producer of processed citrus (Riemenschneider, 1983). However, citrus industry land use is now approaching what it was prior to the severe freezes of the 1980s.

Tree and crop losses were reduced by using various freeze protective methods during many of the freezes that occurred in Florida. Petroleum heaters have been effective in the past, but due to fuel cost and pollution concerns have fallen into disuse (Parsons et al., 1982). Wind machines have also been used during radiative freezes to mix air layers altering the natural temperate gradient during the night within a citrus grove (Turrell, 1973). Windbreaks also offer some freeze protection during advective freezes (Martsolf et al., 1986).

Table 2.1. Summary of severe freezes and their general effects on the Florida citrus industry (adapted from Rogers and Rohli (1991)).

		Citrus Damage				
Year	Dates[z]	Fruit	Mature Trees[y]	Young Trees[y]	Economic Damage Reported / Notes	source[x]
1766	3 Jan	Yes				Fairbanks, (1895)
1835	7-8 Feb	Yes	++	++		Fairbanks (1895)
1880	30 Dec	Yes				
1886	10-12 Jan	Yes	+	+++	3 million $	Mitchell (1917)
1894	28-29 Dec	Yes			86-129,000 mt fruit	
1895	8-9 Feb		+++	+++	Trees deacclimated due to warm temperatures	Wilder (1948)
1899	12-13 Feb		+	++	Trees deacclimated due to warm temperatures	Abbe (1899)
1905	26-27 Jan	Yes		+		Garriott (1905)
1909	30 Dec	Yes				Von Herrmann (1909)
1917	3-5 Feb	Yes	++	+++	Trees killed back to soil banks	
1928	3-4 Jan	Yes				
1934	12-13 Dec	Yes	++	+++		
1940	28-29 Jan	Yes			Trees freeze hardy due to drought induced dormancy	
1947	10-11 Feb	Yes	+	+++	50 million $	
1957	12-13 Dec	Yes	++	+++	over 100 mt fruit	O'Connor (1958)
1962	9-13 Jan		++	++		Starke (1962)
1962	13-14 Dec	Yes	+++	+++	500 million $ over 2 million mt fruit lost 7-10 million trees killed	Hearn et al., (1963)
1977	19-21 Jan	Yes	+	++		
1981	13-14 Dec	Yes	++	+++	1.3 million mt fruit	
1982	11-12 Jan	Yes	++	++		
1983	25-26 Dec	Yes	+++	+++	2.2 million mt fruit lost many trees killed	
1985	21-22 Jan	Yes	+++	+++		
1989	24-25 Dec	Yes	++	++	20% crop reduction	

[z]When two dates are given minimum temperature typically occurred before dawn on the second day.
[y]+ = leaf drop, ++ = leaf drop and stem damage, +++ = leaf drop, stem damage, tree death
[x]Unreferenced descriptions provided by Rogers and Rohli (1991) from *Weekly Weather and Crop Bulletin*.

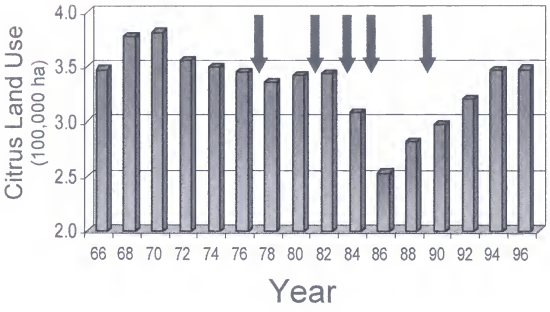

Figure 2.1. Total commercial citrus hectarage over the past three decades. Values indicated include area planted in orange, grapefruit, and specialty citrus fruit. Data from Commercial Citrus Inventory (1996). Arrows indicate severe freezes.

Soil banking is effective in protecting the base of young trees, but is costly, labor intensive, and may damage trees (Jackson et al., 1983). Tree wraps of several types, including corrugated plastic sleeves, shade cloth, polyvinyl covers and nylon fabric have also been used to protect the trunks of young citrus trees (Jackson et al., 1983; 1986). Tree wraps by themselves provide less freeze protection than soil banks (Jackson et al., 1981; Rieger et al., 1988).

Prior to the mid-1980s most growers used overhead sprinklers for freeze protection. This method worked well when freezing temperatures lasted only a few hours, but during long term freezes ice build-up caused limb breakage on trees and, in addition, during advective freeze conditions low sprinkler output often resulted in evaporative cooling which increased freeze damage (Neff, 1996). Water is becoming the most widely used method of citrus freeze protection in Florida (Ferguson and Taylor, 1993). A more recent alternative to traditional freeze protection practices is the use of microspinkler irrigation (Rieger et al., 1986). Again, only the lower portion of the trunk is protected and during severe freezes much of the upper portion of the tree may be killed (Parsons et al., 1985). However, young tree microsprinkler irrigation protection results in a net time loss to growers of only 6 months (Parsons et al., 1985; Rieger et al., 1986), a minor economic loss compared to the time and money required for tree replacement. Moreover, the combination of trunk wraps and microsprinkler irrigation has proven effective in reducing freeze damage in both advective and radiative freezes (Rieger et al. 1985; Rieger et al., 1986; Rieger et al., 1988). The combination of tree wraps and microsprinkler irrigation for freeze protection

is used by 40% of all growers for young citrus tree protection in Florida (Ferguson and Taylor, 1993). There has also been recent interest in elevated and scaffold branch irrigation protection during freezes. More complete protection of citrus trees during freezes has been obtained by moving microsprinklers 1 to 1.3 m into the canopy of mid-sized citrus trees (Bourgeois and Adams, 1991; Parsons et al., 1991).

These freeze protection methods are not without potential drawbacks. Pump systems driving microsprinkler irrigation systems require a reliable back up power source, such as diesel generators, due to frequent brownouts that may occur during freezes. The practice of soil banking has high labor costs and is not used on a regular basis because mechanical removal of soil following a freeze may cause injury to trees. Wind machines are less effective in Florida than in Mediterranean-type climates where radiative freezes are often more prevalent. In summary, Yelenosky (1985) stated that "freeze protection methods are extremely valuable in the short term, but are very sensitive to changing economic conditions" (p. 204).

Citrus Freeze Acclimation

Citrus trees can be injured by temperatures of –2.2 °C and below during moderate freezes, although, depending on species and cultivar, they can withstand lower temperatures without injury (Yelenosky, 1985). Based on numerous seedling studies and field observations on commercially produced

species, mandarins (*C. reticulata* Blanco) are the most freeze hardy. *C. sinensis* (L.) Osb. (sweet orange) and *C. paradisi* Macf. (grapefruit) are the next most freeze-hardy species followed by the most freeze sensitive species, lemons (*C. limon* (L.) Burm f.) and limes (*C. aurantifolia* Christm. Swingle) (Cooper, 1963; Furr et al., 1966; Young, 1963).

In Florida, virtually all commercial citrus trees are budded on rootstocks. For example, 'Hamlin' orange grafted onto either *P. trifoliata* or *C. jambhiri* will produce characteristic orange fruit, but fruit quality, growth, and freeze tolerance will vary. Notably, rootstock selection only has a minor effect on freeze hardiness (Hearn et al., 1963). Even an extremely hardy rootstock choice like *P. trifoliata* needs the appropriate environmental stimuli (at least two weeks of low temperatures) to increase freeze hardiness of the scion (Ziegler and Wolfe, 1961). In contrast, Yelenosky (1992) reported that freeze damage to 'Valencia' orange budded on *P. trifoliata* or 'Etrog' citron (considered very freeze sensitive), was not significantly different following 4 weeks of artificial acclimation (two weeks of 20/10 °C day/night temperatures followed by two weeks of 15/4.4 °C) and exposure to –6.7 °C for 4 hours. This research suggests that current understanding of increases in scion freeze hardiness provided by rootstocks is limited.

Freezing Resistance Mechanisms in Citrus

Avoidance mechanisms. Plants may survive freezing by either tolerance or avoidance mechanisms (Levitt, 1980). The major freeze avoidance mechanism in citrus is supercooling. However, supercooling does not occur to the magnitude that it does in temperate woody crops, which often supercool to –40 °C (George et al., 1982; Yelenosky et al., 1985). In Florida, minimum temperatures are higher on average by about 10 °C than areas where temperate species proliferate (USDA Plant Hardiness Zone Map), so the level of supercooling displayed by temperate crops is not necessary for survival of citrus. Supercooling has the potential to be the most important mechanism in citrus tree survival. Supercooling occurs at varying levels throughout the citrus tree. Maximum supercooling occurs in stems with only limited supercooling possible in flowers (Yelenosky, 1988). In many citrus cultivars minimum supercooling temperatures approach minimum survival temperatures. For example, Yelenosky (1990) found that 'Hamlin' orange trees on rough lemon rootstock exposed to a 12 h photoperiod, 15.6 °C days, and 4.4 °C nights were freeze hardy to –6.7 °C, without damage to stems and leaves; this is thought to be the maximum freeze hardiness level for *C. sinensis* (Yelenosky, 1985). Exotherms for 'Hamlin' orange stems between –4.9 °C and –6.2 °C produced during controlled freezing tests suggests that supercooling occurs to these minimum temperatures (Yelenosky, 1977). Similar minimum values for supercooling were observed for 'Valencia' orange (Yelenosky, 1976b). Since these values are

similar for both cultivars and are within a degree or two of maximum hardiness levels, it appears that supercooling could provide a significant amount of citrus freeze tolerance. However, under field conditions the level of supercooling is more unpredictable and the presence of certain ice-nucleating-agents (INA) may further complicate the picture (Lindow et al.,1978; Yankofsky, 1981; Yelenosky, 1983). Bacteria with INA activity can cause ice nucleation at –2.5 °C negating the freeze protection that supercooling provides (Lindow et al., 1978; Yankofsky, 1981). However, the importance of INAs in citrus freeze tolerance have been questioned recently. Supercooling occurs in all tissues of a citrus tree and, more importantly, depth of super-cooling increases with low temperatures and drought stress (Yelenosky, 1979; Young and Peynado, 1965). This suggests that cultural practices such as irrigation scheduling could be altered to enhance citrus freeze hardiness.

Tolerance mechanisms. Supercooling typically fails as a form of freeze avoidance above –6.7 ° C in laboratory tests (Yelenosky, 1985). Based on the appearance of water soaking, ice nucleation usually starts in the leaves near the midvein and then moves outward towards the margins and into the stem (Yelenosky, 1985). Ice can then propagate from branch to branch through the stems (Lucas, 1954). In controlled freezing tests Yelenosky (1975) found that ice spread at rates of up to 74 cm / min in unhardened 2-year-old 'Marsh' grapefruit trees. Remarkably, freeze acclimation of these trees reduced the rate of ice propagation to one third of the level found in unhardened trees. Once ice

has formed and begun to propagate, injury can often occur (Yelenosky and Horanic, 1969) within minutes (Yelenosky 1976b), although citrus tissues tolerate some level of intercellular ice formation based on young tree recovery data (Yelenosky, 1976b).

Supercooling along with intercellular ice formation and tolerance constitute major mechanisms that citrus trees have to withstand freezes, but what processes are involved in citrus freeze acclimation? Yelenosky (1985) described them clearly when he stated that "although these natural mechanisms are not well understood, they apparently involve adaptive changes in citrus physiology and metabolism that result in changes in cellular composition and physical relationships" (p. 215).

Freeze Acclimation in Citrus

With appropriate cool temperatures citrus trees enter a nonapparent growth period (Yelenosky, 1977; Young, 1970). Concomitantly, multiple physiological changes begin to take place that increase freeze hardiness (Yelenosky, 1985). The development of freeze hardiness during the exposure to low temperatures is known as freeze acclimation. Likewise when citrus trees are exposed to warm temperatures they show a marked decrease in freeze hardiness (Yelenosky, 1985). The process of dehardening, which is often associated temporally with regrowth, is called deacclimation.

Table 2.2. Some cellular changes associated with increases in freeze hardiness.

Cellular change	Notes	Reference
[PEPCase] ↑		Vu and Yelenosky, 1993
anthocyanins ↑	putative	Chap 5 data
carbohydrates ↑	Especially between 15-5 °C	Yelenosky and Guy, 1977
CO_2 exchange rates ↑		Vu and Yelenosky, 1993
free proline ↑	possible membrane protectant	Yelenosky, 1978
reduced glutathione ↑	occurs at low non-freezing temperatures	Guy and Carter, 1982
sucrose ↑	can result in dehydration which leads to more hardiness	Yelenosky, 1978
sugar:starch ↑		Yelenosky, 1978
unsaturated lipids ↑		Nordby and Yelenosky, 1984

Many physiological changes have been associated with citrus freeze acclimation (Table 2.2). These changes also occur in other plant species, but are correlative and not causative with the exception of the increase in unsaturated lipid concentration. Steponkus (1988) used protoplast fusion to show that the presence of mono- and di-unsaturated lipids were critical for plasma membrane freeze acclimation in rye. Anderson et al. (1983) also found that freeze-acclimated citrus leaves survive freezing of a greater proportion of cellular water as compared to non-acclimated leaves and that citrus displayed non-ideal equilibrium freezing.

In addition there are many changes in gene regulation associated with freeze acclimation (Weiser, 1970). Cai et al. (1995) cloned the first six cold-acclimation associated cDNAs from *P. trifoliata,* two of which are similar to group 2 late embryogenisis associated (LEA) proteins. Interestingly, COR19 expression correlated with freeze acclimation, but was down regulated during drought stress. Drought stress (Yelenosky, 1979) and low temperatures (Young, 1970) both increase the freeze hardiness of citrus. Work by Cai et al. (1995) suggests that at least in the case of COR19 there may differences in freeze acclimation based on environmental stimuli.

For the above putative freeze acclimation mechanisms to take place in citrus, the appropriate environmental conditions must exist. Many environmental factors have been implicated in freeze acclimation including light (Yelenosky, 1971) and water relations (Yelenosky, 1979). Temperature, however, is the most

important factor in determining the initiation, termination, and level of freeze acclimation in citrus (Rouse and Wiltbank, 1972; Yelenosky, 1978).

It is clear, in addition to the major factors of drought and low temperature, that cultural practices also can play a critical role initiating and maintaining freeze acclimation and thus improving freeze hardiness in citrus trees. Cultural practices that may have an effect on citrus freeze acclimation include soil management, maintaining air drainage, tree spacing and orientation, pruning, pest and disease control, mineral nutrition, and irrigation. Krezdorn and Martsolf (1984) hypothesized that implementation of these practices might improve freeze hardiness by 1-2 °C. Small, seemingly insignificant, changes in freeze hardiness, such as 1-2 °C, can often mean the difference between little damage and an economic catastrophe.

Effect of Nutrition on Citrus Freeze Hardiness and Acclimation

The effect of nutrition on citrus freeze hardiness has been examined on numerous occasions, with variable, sometimes contradictory, conclusions. Historically, citrus trees have been evaluated following a freeze to determine if there was any correlation between various macronutrient and micronutrient levels and freeze damage. In these cases, only inferences about freeze hardiness, not acclimation, can be made. Lawless (1941) found that the amount of fruit and leaf damage and deadwood pruned (based on cropload) was greatly increased if trees were deficient in Zn and Cu. Spencer (1958) found that applications of

phosphate to 'Ruby Red' grapefruit increased susceptibility to freeze injury. Deficient N and excessive K levels increased injury following a freeze (Koo, 1985), suggesting that maintaining leaf nutrient concentrations within optimum ranges increases freeze hardiness.

In contrast, other researchers such as Smith and Rasmussen (1958) and Krezdorn and Martsolf (1984), have concluded that non-deficient nutrient levels have little effect on citrus freeze hardiness. In order to address problems associated with post freeze evaluations, Maurer and Davies (1994) determined electrolyte leakage and leaf killing points on young 'Redblush' grapefruit trees throughout the winter. They reported that leaves with low N concentrations (2.0 %) were less hardy than leaves with higher N levels. These data again suggest that nutrient concentrations in the recommended range will have little effect on the level of citrus leaf freeze hardiness; however, nutrition may have an effect on acclimation.

Smith and Rasmussen (1958) found that discontinuing fertilizer application in Aug rather than the end of Oct resulted in significantly less leaf and stem freeze damage in 1956-57 on one-year-old citrus trees. Significantly, the effect was observed regardless of application rate and whether or not a complete fertilizer or only ammonium nitrate was used. Conversely, early termination of fertilizer application had no effect on the same trees during a freeze that occurred the following year. Thus, the dogma exists that fertilization of citrus trees should stop in the fall to prevent late season growth flushes (Hume, 1956) that are freeze-sensitive (Young, 1970).

Historically, rapid deacclimation during February was detrimental for young citrus trees following the freezes of 1895 (Wilder, 1948) and 1899 (Abbe, 1899) (Table 2.1). Interestingly, Maurer and Davies (1994) found leaves with high N concentrations (3.6 – 3.8% N) were faster to deacclimate during the spring than leaves with N levels in the normal range. However, it is likely that citrus trees acclimate and deacclimate based on air and soil temperatures (Rouse and Wiltbank, 1972; Wilcox et al; 1983; Young and Peynado, 1965), rather than on tree nutrition. Temperature fluctuations that occur in Florida may cause citrus trees to acclimate and deacclimate several times during the winter (Wiltbank and Oswalt, 1983; Maurer and Davies, 1994). These changes in acclimation level are observed routinely regardless of nutrition management programs.

In summary, cultural practices related to nutrition can have varying effects on freeze acclimation, deacclimation, and thus hardiness. However, the exact role nutrition management plays is unclear. Therefore, studies controlling for optimum nutrition levels, fertilizer application frequency, and fall fertilization termination date are needed to determine if any changes in fertilizer application methods can be made that make positive changes in acclimation and deacclimation patterns and increase citrus freeze hardiness.

Effects of Irrigation Scheduling on Citrus Freeze Hardiness

Another cultural practice that may affect freeze hardiness and acclimation of citrus trees is irrigation scheduling. In 1939, a particularly dry fall resulted in drought-stressed trees which withstood a January 1940 freeze very well, suggesting anecdotally that the water deficit imparted increased freeze hardiness (Rogers and Rohli, 1991). Cooper (1964; 1965) also reported that reducing irrigation levels in the fall and winter not only increased freeze hardiness, but improved "dormancy" induction. In contrast, Koo (1981) observed that irrigated 'Hamlin', 'Valencia', and 'Pineapple' orange trees had better survival than non-irrigated counterparts. Reducing irrigation to the point of a dehydrative stress increased freeze hardiness for containerized 'Valencia' orange and 'Star Ruby' grapefruit (Yelenosky, 1975; Yelenosky, 1979). Davies et al. (1981) also found that moderate water stress during the fall and under-tree sprinkling for freeze protection resulted in reduced fruit loss and leaf damage 6 weeks following a radiative freeze. However, the problem with these studies was that irrigation levels were out of the optimum range for tree growth and fruit production. There were also the other mitigating factors of canopy density and general tree health which could have contributed to increased freeze hardiness. Marler and Davies (1990) found that irrigation at sub-optimal levels reduced growth. This type of irrigation schedule has the potential to produce increased freeze acclimation in citrus trees, but reductions in growth are economically unacceptable. Limited

research has examined the effects induced by altering irrigation scheduling in the recommended optimum range.

Breeding Citrus for Improved Freeze Hardiness

Another potential method of increasing freeze hardiness in commercial citrus is through breeding (Young et al., 1982). Fortunately, citrus is capable of some degree of interspecific and even intergeneric hybridization. This results in many possible hybrids such as lemonages, lemonimes, lemandarins, tangelos, and many others (Davies and Albrigo, 1994). Citrus also forms wide hybrids with the remaining five genera in the true citrus group which includes *Fortunella*, *Poncirus*, *Eremocitrus*, *Microcitrus*, and *Clymenia* (Barrett, 1985). However, all current commercially important citrus cultivars and rootstocks belong to three genera; *Citrus*, *Fortunella*, and *Poncirus* (Swingle and Reese, 1967). The great diversity among the five genera (although there is taxonomic debate about the current classification system) of true citrus results in the possibility of integrating many advantageous characteristics, such as freeze hardiness and citrus Tristeza virus (CTV) resistance, into scion and rootstock breeding lines and ultimately into cultivars earmarked for commercial release (Barrett, 1985; Hearn, 1994). Such crosses have been made in the search for improved freeze-hardy rootstocks for over 100 years and were initiated following the freezes of 1894 and 1895 (Swingle and Reese, 1967). Those initial efforts have evolved to current genetic

studies which include the mapping of the citrus genome for quantitative trait loci (QTL)s associated with freeze hardiness (Cai et al., 1994).

Among these genera *Poncirus* is the only truly deciduous genus and *Eremocitrus* the only true xerophyte (Barrett, 1985). Barrett (1977; 1981; 1985; 1990a; 1990b; 1994) has worked extensively in the selection of citrus with appropriate traits for recombination and the production of both intrageneric and intergeneric hybrids at the USDA in Orlando, FL. He has used four genera, *Eremocitrus*, *Fortunella*, *Microcitrus*, and *Poncirus,* in crosses with many different citrus species (Barrett, 1985). *Eremocitrus* was used as a trait source for tree freeze hardiness, very early fruit maturity, and a pronounced quiescent period. *Fortunella* was hybridized with *Microcitrus papuana* for the purpose of producing genetic bridges. *Microcitrus* and *Poncirus* were also hybridized in hopes of producing an improved rootstock that would combine the traits of burrowing nematode and *Phytophthora* resistance from *Microcitrus* with the resistance to citrus nematode, citrus tristeza virus (CTV), and freezing of *Poncirus*. In addition, *Poncirus* was used as a parent with other *Citrus* species as a source for freeze hardiness, deciduousness, and CTV resistance for scions.

As a result of several crosses, Barrett (1990) released 'US 119' ([*Citrus paradisi* Mac. cv. Duncan X *Poncirus trifoliata* (L.) Raf.], for use as an intergeneric hybrid citrus scion breeding line. It yielded negative enzyme-linked immunosorbent assay (ELISA) readings over several years after being inoculated with five Floridian strains of CTV (Garnsey et al., 1981, 1987). In addition, it survived the 1981, 1983, and 1985 freezes (Barrett, 1990) which killed many

commercial citrus trees and virtually eliminated citrus production in the northern ridge area of central Florida. It was also found to have fruit qualities similar to a sweet orange and the "unusual combination of fine-textured and very firm flesh" (Barrett, 1990). Overall the fruit is palatable, but does not have high enough quality to replace any existing commercial sweet orange cultivars.

Selection 17-11 (*C. grandis* cv. North East by [*Citrus paradisi* Mac. cv. Duncan X *P. trifoliata* (L.) Raf.]) has not been officially released and is another selection by H.C. Barrett (Wayne Sherman, personal communication). Nothing has been reported about this genotype, but it has promise of having superior freeze hardiness (Wayne Sherman, personal communication). Its canopy is somewhat more sparse than that of 'US 119' growing under similar conditions and has thorns which continue to elongate for some time (up to 20 cm). 'US 119' and selection 17-11 have a great deal of potential as scion breeding lines, but a more thorough investigation of their freeze hardiness and acclimation rates is needed.

Early Events in Citrus Acclimation

In order to control for numerous variables in natural freeze acclimation of citrus, many researchers have long turned to studies that utilize artificially controlled freeze acclimation in their studies on citrus freezing tolerance (Yelenosky 1975; 1978; 1979; 1990; 1994; Young 1969, Young and Peynado 1967) (see Table 2.3). A variety of acclimation protocols have been used to

Table 2.3. Various temperature and daylength regimes used to freeze-acclimate citrus.

Reference	Year	Day/Night Acclimation Temps (°C)[z]	Day-length (h)	Interval data collected (days)	Data collected[y]
Young	1969	21/10, 15.6/4.4, 10/-1.1, 7.2/-3.3	12	14	leaf hardiness, sugar content
Young and Bell	1974	15.6/4.4, 10/-1.1	12	42	photosynthesis rate, moisture levels
Young and Mann	1974	15.6/4.4	12	28	light and electron microscopy of frozen and unfrozen cells
Yelenosky	1975	21.1/10, 15.6/4.4	12	28	stem electrical resistance
Yelenosky	1978	10 /10	24	7	stem and leaf kill
Wilcox et al.	1983	20/10/15/5	12	14	Transpiration, diffusive resistance, leaf xylem potential, leaf relative water content, etc.
Yelenosky	1990	15.6/4.4	12	35	sucrose and starch content, amino acid concentration, stem and leaf dieback
Yelenosky et al.	1993	21/10, 15/4	12	28	leaf and stem injury

[z]When multiple temperature pairs are given typically plants were exposed to each temperature for 14 days.
[y]In many cases more parameters were measured than shown.

artificially freeze acclimate citrus. Acclimating day/night temperatures ranged from a high of 21/10 °C to a low of 7.2/-3.3 °C. Sometimes freeze acclimation program temperatures were changed in the course of the experiment (Young, 1969), usually biweekly, and on other occasions acclimating temperatures were held constant during the day and night (Yelenosky, 1978). In addition, data were collected at intervals of a minimum of 7 days (Yelenosky, 1978) to a maximum of 42 days (Young and Bell, 1974). In summary, there is not a standard acclimation protocol that has come to be accepted in freeze hardiness studies and data collection intervals are large. Similarly, field studies, where temperatures and rainfall vary from year to year, have examined changes in citrus acclimation weekly (Wiltbank and Oswalt,1983), or biweekly (Maurer and Davies, 1994). The logistics of field sampling for freeze acclimation determination are difficult and actually estimate leaf, not whole tree hardiness. No information is available on rapid changes associated with the initial hours of exposure to low temperatures.

Besides temperature, effects of light and photoperiod on freeze hardiness have also been examined. Yelenosky (1971) showed that light is required to attain maximum freeze hardiness; in the absence of light there would be no photosynthesis and possibly a reduction in sugar accumulation, which has been correlated with increased freeze hardiness. In addition to the need for light, photoperiod also plays a definitive role in the growth of citrus relatives. Warner (1979) also found that an 8 h SD photoperiod could significantly slow growth of several *P. trifoliata* cultivars based on stem diameter, linear growth, and branch number. This was significant since researchers have conducted artificial

acclimation studies on citrus using a neutral photoperiod of 12 h (Table 2.3). Apparently, 8 h daylength alone also reduced leaf defoliation and twig damage in 'Redblush' grapefruit without exposure to low temperatures (Young, 1961). Perhaps it would be more appropriate to use a long day (LD) photoperiod of 16 h to ensure separation of effects associated with exposure to low temperature from those associated with possible SD induced quiescence and freeze hardiness. Although it is quite possible that some responses to low temperature and abbreviated photoperiod work in conjunction to provide maximum freeze hardiness.

Determining Citrus Freeze Hardiness

In order to study freeze acclimation, deacclimation, and hardiness, it is necessary to have a reliable measure of freeze hardiness. Hutcheson and Wiltbank (1970) modified the leaf freezing point (LFP) method of determining freeze hardiness for different orange cultivars based on early reports by Jackson and Gerber (1963). This method involved using detached leaves with an attached thermistor lowered into a freezer. LFPs were repeatable, but have several shortcomings, the most obvious being that freezing of a leaf is not necessarily correlated to physiological damage because citrus leaves tolerate intercellular freezing during the appropriate conditions. Moreover, this technique only estimates leaf damage which may or may not be correctly extrapolated to stem and trunk damage during a freeze.

Regrowth, which is difficult to evaluate objectively in some cases, is another measure of freeze hardiness. It has been used extensively (Yelenosky, 1971; 1975; 1976a+b; 1992; Young and Peynado, 1967) for seedling and young trees during controlled freezes and on citrus in the field following a naturally occurring freeze (Bourgeois and Adams, 1991; Cooper et al., 1962; 1963; Hearn, 1963; Lawless, 1941). However, this method can have drawbacks. Hardiness is defined as the extent of leaf, stem, and trunk death. Regrowth in the field following a freeze, although related to the extent of injury, may be a function of scion, rootstock, or general health of the tree. For example, similarly damaged 'Valencia' trees budded on rough lemon will regrow faster following a freeze than those on sour orange.

The best method of determining freeze hardiness is to evaluate leaf drop and stem and trunk dieback directly. Freeze damage values have often been presented as temperatures at which 50% of leaves died (LT_{50}) based on the appearance of watersoaking (Young, 1969;1970). At other times data were presented as percent leaf kill, stem dieback, or rootstock dieback (Yelenosky, 1975; 1979). Data taken by visually observing damage to stems and leaves can be less accurate due to human error than other more physiological measures of freeze hardiness.

Wiltbank and Oswalt (1983) used a method derived from techniques utilized to determine freezing tolerance of wheat and potato plants (Marcellos et al., 1979; Sukumaran and Weiser, 1972a; 1972b). They determined leaf killing points (LKP) by subjecting 18 excised citrus leaves (3 per temperature) to 6

sequentially lower freezing temperatures at intervals of 1 °C. Electrolyte leakage (EL) was determined for samples at each temperature and a sigmoid curve fit to the data. The inflection point of the curve was associated with the LKP. The LKPs were based on either connected or fitted lines which often resulted in arbitrary or interpolated values. Also the LKP was not correlated with actual leaf death. Nevertheless, LKP values determined by this method correlated well with actual freeze damage observed on trees during the 1981 and 1983 freezes.

Maurer and Davies (1994) measured hardiness using LKPs based on EL at –3 to –7 °C in a very similar manner to that of Wiltbank and Oswalt (1983), but also visually observed a second set of leaves 5-7 days following the controlled freeze test which compared EL values to leaf mortality. Both methods of LKPs were in close agreement throughout their study. Another possible problem with this technique was the reported thawing temperature of 25.6 °C. Rapid thawing is known to cause additional damage to frozen tissues in some cases (Levitt, 1980). However, citrus leaf temperatures in the field often increase from –6.7 to 10 °C almost immediately when the sun rises, yet there is no evidence that this causes additional damage (Davies, personal communication).

Using unmodified EL data might also be a good way to determine citrus freeze hardiness and acclimation (by measuring over several dates). Methods based on raw EL data have been useful in freeze evaluations of alfalfa (Dexter et al., 1932), strawberry crowns (Flint et al., 1967), and St. Augustine grass (Maier et al., 1994). Direct EL measurements provide an estimate of cell membrane disruption and extent of cell damage. Sources of error are greatly reduced by not

fitting lines to the data. However, freeze tests on whole plants must be used to calibrate EL damage with whole plant freeze recovery.

Summary and Research Directions

There has been much time and money invested in both basic and applied research on many aspects of citrus freeze hardiness and acclimation in Florida during the last century. As a result there is some understanding of how citrus responds physiologically before, during and after a freeze. We also know the value and limitations of many different freeze protection measures for citrus trees. In addition, breeding efforts are well underway that hope to bring improved freeze hardiness traits into citrus cultivars of commercial value. However, this body of research is incomplete and there is still much to be learned about citrus freeze hardiness, acclimation, and deacclimation.

Hope for more reliable freeze protection for the Florida citrus industry could potentially lie in three directions away from traditionally researched methods. First, the effect of cultural practices such as irrigation scheduling and fertilizer regimens on citrus freezing acclimation is not well understood. Secondly, advanced, potentially hardier citrus scion breeding lines need to be evaluated for field performance and acclimation-deacclimation patterns. Finally, many thorough studies researching controlled freezing acclimation and acclimation under natural field conditions have been completed, but typically data are evaluated at time intervals of days and weeks, even months. Research

concerning short term changes associated with citrus freeze acclimation has not been conducted and would provide valuable basic information on citrus freeze acclimation.

CHAPTER III
IRRIGATION SCHEDULING AND NUTRIENT APPLICATION FREQUENCY EFFECTS ON FREEZE ACCLIMATION AND GROWTH OF 'HAMLIN' ORANGE TREES IN FLORIDA

Introduction

Severe freezes have lead to repeated tremendous economic losses to the Florida citrus industry in the 1980s. Over 1 billion dollars in damages resulted from the freezes that occurred in Florida from 1981 to 1985. In some of these freezes tree and crop losses were reduced by using freeze protection methods such as petroleum heaters, soil banking, tree wraps, and microsprinkler irrigation (Davies et al., 1984; Jackson et al., 1983; Parsons et al., 1982). Although these methods, particularly microsprinkler irrigation in conjunction with tree wraps, are effective for protecting young trees, water availability and cost may limit their use (Rieger et al., 1985; 1986).

An alternative method for reducing economic losses to young citrus trees during freezes is to obtain a better understanding of how irrigation and nutrition affect freeze hardiness and acclimation. Past reports suggest that increasing water stress will make many plants including citrus more freeze resistant (Husain and Cooper, 1958; Yelenosky, 1979). Similarly, Davies et al., (1981) observed that withholding irrigation in the fall increased hardiness of mature 'Orlando'

tangelo trees. However, irrigation effectiveness for increasing freeze hardiness in the field has not been consistently demonstrated.

The role of nutrition in freeze hardiness is poorly understood. Some researchers have observed that nutrition plays no role in citrus freeze hardiness (as reviewed by Krezdorn and Martsolf, 1984). Other research has shown that deficiencies in Mg (Lawless, 1941) or N (Koo, 1985) increase susceptibility to freezing damage. Based on early research (Hume, 1956; Rasmussen and Smith, 1961), the dogma exists that increasing fertilizer application rate during the growing season and terminating application in the fall, as opposed to applying at lower frequency all year, should help to prevent late season growth flushes that are freeze sensitive. However, temperature probably has a greater influence on late season growth flushes than nutrition or irrigation.

'Hamlin' is the most widely planted sweet orange in Florida, representing 25% of all citrus acreage (Fla. Agr. Stat. Ser., 1996). 'Hamlin' orange trees are widely planted because they have consistently high yields and early maturity (Fla. Agr. Stat. Ser., 1989). Freeze hardiness of young 'Hamlin' trees has been determined by Yelenosky (1990) who found that after 6 consecutive weeks of 15.6 days and 4.4 °C nights stems and leaves survived 4 hours at –6.7 °C with little or no damage. Therefore 'Hamlin' orange trees freeze-acclimate as deeply as any *C. sinensis* cultivar (Yelenosky, 1985).

There is little information on post-freeze fertilization practices for young trees. Davies and Maurer (1990) found that 6-year-old mature 'Hamlin' non-bearing trees had similar growth and appearance when up to 3.4 kg of fertilizer

was applied after two subsequent freezes or when no fertilizer was applied. However, nutrition recommendations for young citrus trees following freeze damage are lacking.

The objective of this study was to determine if reducing fertilizer application frequency and irrigation levels in the fall (based on plant growth flush status) would increase freeze hardiness of young 'Hamlin' orange trees in the field. In addition, the effects of nutrition practices on tree growth and post-freeze recovery were examined.

Materials and Methods

Plant material and experimental design. Commercially grown, containerized 'Hamlin' orange (*C. sinensis* (L.) Osb.) trees on 'Swingle' citrumelo rootstock (*C. paradisi* Macf. X *Poncirus trifoliata* (L.) Raf.) (Rasnake Nursery, Winterhaven, FL) were planted on 4 Apr 1994 on double beds oriented north to south with 7.62 m between beds and 4.0 m between trees. Trees were transported and planted at the University of Florida Horticultural Research Unit in Gainesville, FL on the day of purchase. Trees were irrigated for 2 hours on alternating days for 4 weeks. Irrigation was supplied using 90° 38 liter•hr^{-1} Maxi-Jet™ emitters. Fiberglass trunk wraps (R11) were placed on each tree on 3 May 1994 primarily for freeze protection and to reduce sprouting. Trunk wraps remained for the duration of the study. Trees were weeded by hand 6 times and sprayed using glyphosate once during the 2-year study. The soil type was

Myakka fine sand (loamy, siliceous, hyperthermic, Grossarenic Paludults). This soil has a volumetric capacity of 10.2%, a permanent wilting point of 1.7%, and a 2.77 $g \bullet cm^{-3}$ mean bulk density (Maurer and Davies, 1993).

Statistical analysis. This study consisted of a completely randomized design. Treatments were arranged as a 3 (irrigation scheduling) X 3 (N rates) factorial experiment with 11 individual tree replicates per treatment. All statistical calculations including regression analysis were completed using Statistical Analysis Software (SAS Institute, Cary, NC). A general linear model procedure (GLM) was used to perform analysis of variance (ANOVA). In addition, trunk diameter and plant heights were evaluated using an analysis of covariance to standardize any initial differences in tree sizes. Where analysis of covariance was completed adjusted LSmeans are shown.

Irrigation scheduling treatments and growth flush determination. The three irrigation treatments (Table 3.1.) were based on presence or absence of growth flushes and soil water depletion (SWD) level as determined using a Troxler model 4300 neutron probe (Research Triangle Park, NC). The first treatment was irrigated when SWD fell below 20% regardless of tree growth status (20 SWD); the second treatment was irrigated at 20% SWD through the end of the second flush and irrigated at 45% SWD until the following spring (20-2 SWD). The final treatment was irrigated at 20% SWD through the first growth flush and at 45% SWD until the following spring (20-1 SWD). The 20 SWD, 20-2

SWD, and 20-1 SWD treatments received 28, 28, and 26 two-hour irrigations in 1994 and 20, 17, and 12 two-hour irrigations in 1995, respectively (Table 3.1). Soil water depletion values and irrigation duration time in this experiment were found to be in the optimum range for 'Hamlin' on sour orange rootstock planted on this site (Marler and Davies, 1989). Probe tubes for each irrigation treatment were randomly placed 15-cm from the base of a different tree. Irrigation scheduling decisions were based on SWD calculated from the mean soil moisture content of 5 tubes for each treatment. Probe readings were taken weekly, but the duration between readings varied depending on rainfall. About every week during the growing season each tree was evaluated for the presence or absence of a growth flush. For the tree to be scored as flushing there had to be rapidly elongating vegetative buds or expanding leaves present on at least three different branches, thus avoiding isolated bud-break which occurs occasionally on individual shoots. The growth was considered finished when all leaves from the current flush were fully expanded.

Fertilizer Rates and Application Frequency. Fertilizer rates were the same for all treatments, (0.136 kg N/tree) in 1994 and 0.227 kg N/tree in 1995 based on recommendations from Koo (1984). However, amount of N per application varied. Granular fertilizer (8N-2P_2O_5-8K_2O-2Mg, Seminole Fertilizer) was applied biweekly at 5.9 g N (22 X), 10.5 g N (13 X), or 15.1 g N (7 X) in 1994 and 12.6 g N (18 X), 16.2 g N (14 X), or 22.7 g N (10 X) during 1995 (Table 3.2.). Treatments 1, 2, and 3 were applied until 1 Jan, 1 Nov, and 1 Sept, respectively.

Table 3.1. Irrigation scheduling for 1-year-old 'Hamlin' orange trees located at the Horticultural Research Unit in Gainesville, FL, 1994-95.

	1994		1995	
Irrigation regime	Total (no.)	liters/tree/year[z]	Total (no.)	Liters/tree/year[z]
1. 20% SWD for all growth flushes (20 SWD)	28	2121	20	1515
2. 20% SWD for growth flushes 1,2 followed by 45% for remaining flushes (20-2 SWD)	28	2121	17	1288
3. 20% SWD for growth flush1 followed by 45% for remaining flushes (20-1 SWD)	25	1969	12	909

[z]Trees were irrigated for 2 h using 38 liter•h^{-1} Maxi-jet™ emitters.

Table 3.2. Fertilizer frequency and application rate for 1-year-old 'Hamlin' orange trees located at the Horticultural Research Unit in Gainesville, FL, 1994-95.

	1994			1995		
Fertilizer regime	Total N[z] (kg)	No. appl.	N / appl. (g)	Total N[z] (kg)	No. appl	N / appl. (g)
1. Apr. – Dec.	0.136	23	5.9	0.227	18	12.6
2. Apr. – Nov. 1st	0.136	13	10.5	0.227	14	16.2
3. Apr. – Sept. 1st	0.136	9	15.1	0.227	10	22.7

[z]N applied using an 08N-02P_2O_5-08K_2O-02MgO dry granular fertilizer

Plant growth measurements. Plant height was measured from the soil level to the highest apical meristem. Trunk diameter was measured using a caliper oriented north-south. Repeatability was attained by marking the trunk about 50 cm above soil level with paint or permanent ink (typically 2-3 cm above the trunk wrap). Plant height and trunk diameter measurements were taken biweekly or monthly depending on the year.

Freeze hardiness determination. From 11 Nov 1994 to 7 Mar 1995 and 10 Sept 1995 until 11 Dec 1995 leaves from each of the 9 treatment combinations (3 irrigation schedules X 3 fertilizer application frequencies) were collected weekly in 1994 and monthly in 1995. No more than 5 leaves were collected from any individual tree on a given sampling date. Collection took place before 0800 h and leaves were transported in a cooler to the lab. Level of freeze hardiness was determined based on a method developed by Flint et al. (1967). All leaves were washed in three successive baths of distilled deionized water (dd) to remove dust and other field debris. Three leaf discs were removed from each leaf collected and placed in a single 16X100 mm tube, and 100 µl of dd water was added. In 1994 there were 6 replicate tubes (6 leaves) for each of 9 combination of treatments and 4 test temperatures (a total of 216 leaves examined per freeze test). In 1995, 10 replicate tubes (10 leaves) for each treatment and 4 test temperatures were used (a total of 360 leaves examined per freeze test). Tubes were racked and immersed in a pre-cooled (-1 °C) model 2425 glycol bath and circulator (Forma Scientific, Marietta Ohio) and allowed to

equilibrate for 30 min. One subsample of all treatments was removed as a nonfrozen control. Water in the bottom of the remaining tubes was then nucleated homogeneously in all tubes by adding a small cloud of ice crystals. Temperatures in the bath were lowered at 0.1 °C•min^{-1} until –3, -6, or –9 °C was reached. Samples were held at each test temperature for 1 h and then a subsample was removed. Frozen samples were allowed to thaw overnight at 1 °C to eliminate injury caused by rapid warming. Following the thaw, tubes were removed from the incubator, allowed to reach 20 °C, and initial electrolyte leakage (EL) values were taken with a model CDM3 conductivity meter (Radiometer, Copenhagen, Denmark). Samples were autoclaved for 25 min to simulate 100% damage and a second EL measurement was taken. Percent injury for each sample was determined using the equation shown below (F = electrolyte leakage of sample; UFC = electrolyte leakage of nonfrozen control; BA = before autoclaving; AA = after autoclaving) (Flint et al., 1967).

$$\%\ Injury = 1 - \left[\frac{1 - \left(\frac{F_{BA}}{F_{AA}} \right)}{1 - \left(\frac{UFC_{BA}}{UFC_{AA}} \right)} \right] X\ 100$$

This equation compensates for differences in EL due to leaf disc excision, or inherent variation among leaves.

Nitrogen analysis. Ten fully expanded leaves free of disease symptoms and insect, pathogen, or mechanical damage were sampled from 5 different trees (each tree was a replicate) in each of the 9 treatments on 18 Oct 1994 and 2 Oct

1995. All leaves sampled were about same age and from the spring flush as recommended by Koo et al. (1984). Leaves were washed in three successive baths of dd water and dried in an oven at 70 °C for 72 h or until dry. Tissue was then ground to a fine powder using a Wiley mill until it could pass through a 40 mesh screen. Samples were stored in scintillation vials. A 250 mg sub-sample of each sample was placed in a Pyrex digestion tube and 2.5 ml of concentrated H_2SO_4 added. After 24 h a total of 2 ml of H_2O_2 was added. After the reaction stopped tubes were placed on a digestion block and heated to 375 ° C for 1 h. Another 1 ml of H_2O_2 was added and after the reaction stopped tubes were returned to the reaction block for another hour. This was repeated until the samples clarified. Samples were cooled to 23 °C and dd water is added to bring the sample to a total volume of 25 ml. This solution was then passed through a No. 2 Whatman filter paper (Fisher) into a 25 ml scintillation vial and stored at 4 °C. Total N was determined at the Analytical Research Laboratory (Gainesville, FL) by the micro-Kjeldahl procedure (Wolf, 1982) using a Rapid Flow Analyzer (RFA:Alpkem Corp., Clackamas, OR).

Freeze Recovery Study. On 24 Dec 1995 an irrigation failure resulted in all trees being exposed to –9.3 °C air temperature. Two weeks following the freeze trees had nearly 100% defoliation and 10-20% stem dieback. Fertilizer was applied monthly from 3 March 1996 through 7 July 1996 at 12.6 g N, 16.2 g N, or 22.7 g N. Trunk diameters of the scion and rootstock were recorded monthly. All trees were irrigated when SWD rose above 20%.

Results and Discussion

Growth and development. Soil moisture levels for different irrigation treatments for 1994 and 1995 are shown in Fig. 3.1. In 1994 trees had three growth flushes of roughly equal duration lasting about 60 days each (Fig. 3.2.A). The first flush occurred over 9 weeks and average growth flush activity peaked at two different times, during the 4^{th} and 5^{th} week and in the 8^{th} week. This bimodal flushing pattern did not occur during any other time period during the course of the 2-year experiment. There were several periods of heavy rainfall and a period of nights with cool temperatures which may have contributed to this pattern (Fig. A.2). Irrigation regime had a significant effect on the percentage of trees that had growth flushes during the 3^{rd} ($p \leq 0.05$), 6^{th} ($p \leq 0.01$), and 7^{th} ($p \leq 0.01$) weeks. In all cases, irrigation schedules 20-2 SWD and 20 SWD resulted in significantly more trees displaying growth activity than did trees under the 20-1 SWD schedule (Table 3.3). In 1994 the second growth flush began immediately after the first and lasted 9 weeks. Irrigation regime again had a significant effect on % trees actively flushing in the 2^{nd} ($p \leq 0.05$), 3^{rd} ($p \leq 0.001$), and 4^{th} ($p \leq 0.01$) weeks. The 20-1 SWD treatment had 50% less trees flushing than the 20 SWD treatment during the 2^{nd} week of flush 2 and up to 50% less than trees under irrigation schedules 20 SWD and 20- 2 SWD during the 3^{rd} and 4^{th} weeks (Table

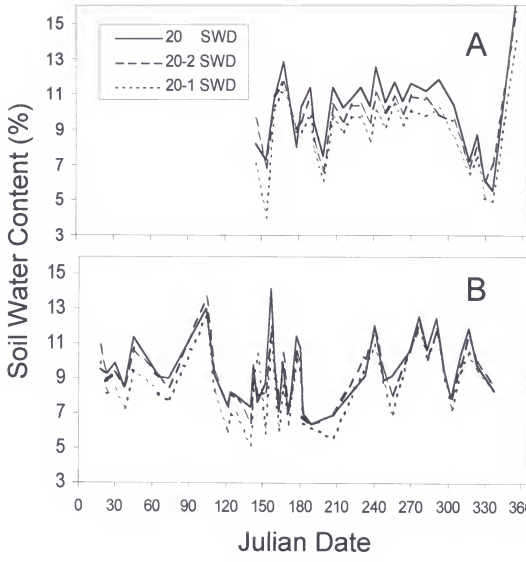

Figure 3.1. Mean soil water content (%) at the Horticultural Research Unit in Gainesville, FL in 1994 (A) and 1995 (B) related to irrigation treatments. Soil water content was determined by taking readings at a depth of 0.6 m with a neutron probe from randomly placed probe tubes throughout the experimental block for each irrigation treatment, n=5.

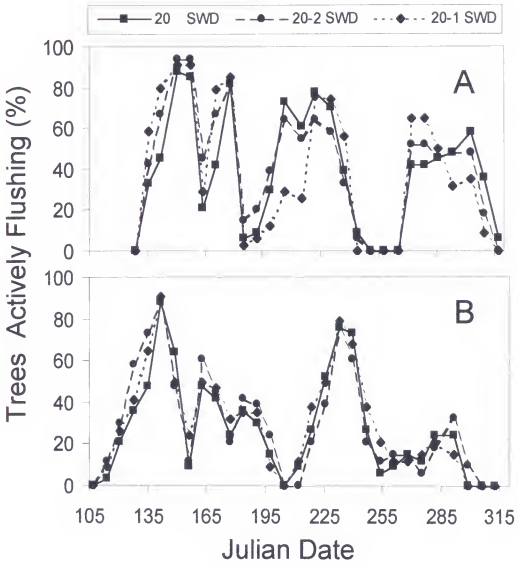

Figure 3.2. Irrigation scheduling effects on growth flushes of young 'Hamlin' orange trees at the Horticultural Research Unit in Gainesville, FL in 1994 (A) and 1995 (B). Trees were examined weekly for the presence or absence of a rapidly expanding vegetative buds on at least three different branches. Individual trees were considered flushing until leaves were fully expanded. Mean % flushing trees shown, n=33.

Table 3.3 Significant effects of irrigation regime on 1-year-old 'Hamlin' orange tree mean % growth activity in 1994 at the Horticultural Research Unit in Gainesville, FL.

Irrigation	Growth Flush[z]						
	1			2			3
Regime	week 3	week 6	week 7	week 2	week 3	week 4	week 6
SWD	45.3b	21.2b	42.4b	30.3ab	72.7a	60.6a	36.4a
SWD-2	66.7ab	45.5a	66.7a	39.4a	63.6a	54.6a	18.2ab
SWD-1	79.4a	29.4ab	79.4a	11.8b	29.4b	26.5b	8.8b

[z]Letters represent groupings of means based on Duncan's multiple range test performed on arcsine transformed data, same letters not significantly different, $p \leq 0.05$, n=33.

3.3). There was a 3-week-period of no growth activity between the 2nd and 3rd flushes. Different irrigation regimes had no significant effect on tree growth activity during the 3rd growth flush of 1994.

In 1995 there were 4 growth flushes, 3 major flushes and a fourth minor flush when no more than a 35% mean flushing activity was recorded (Fig. 3.2.B). Growth started 3 weeks earlier in 1995 than in 1994 and the 3 major flushes were finished more than a month before they were in 1994. In contrast to 1994, there was also only one period during the growing season when % trees actively flushing reached zero. Irrigation scheduling had no significant effect on % of trees flushing at any date in 1995.

Fertilization application frequency had no significant effect on % trees flushing in 1994 and 1995.

Neither irrigation scheduling or fertilizer application frequency had a significant effect on trunk diameter in 1994 (Fig. 3.3.A). Trunk diameter among all trees increased from a mean of 7.8 to 16.8 mm. In 1995 probability values from the analysis of covariance suggested that irrigation might play a significant role in trunk diameter growth. However, slopes of lines produced using regression analysis were not significantly different based on t-tests (Fig. 3.3.B). Mean trunk diameter for all trees increased from 21.9 to 26.9 mm in 1995.

Neither N application rate (Fig. 3.4.A) or irrigation scheduling treatments had a significant effect on tree height in 1994. Mean plant height across all treatments increased from 81.8 initially to114.1 cm at seasons end. Based

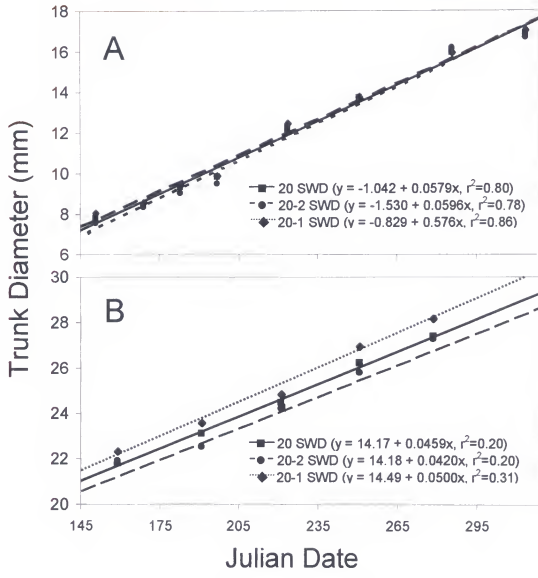

Figure 3.3. Effects of irrigation scheduling on trunk diameter of young 'Hamlin' orange trees at the Horticultural Research Unit in Gainesville, FL in 1994 (A) and 1995 (B). Analysis of covariance was completed for each date shown. There was no significant difference in trunk diameter based on LSmeans analysis ($p \leq 0.05$) for any date shown, although irrigation did add significantly to the covariance model for most dates in 1995. Slopes of individual regression individual lines were not significantly different in 1994 or 1995. Adjusted LSmeans are shown, n = 33.

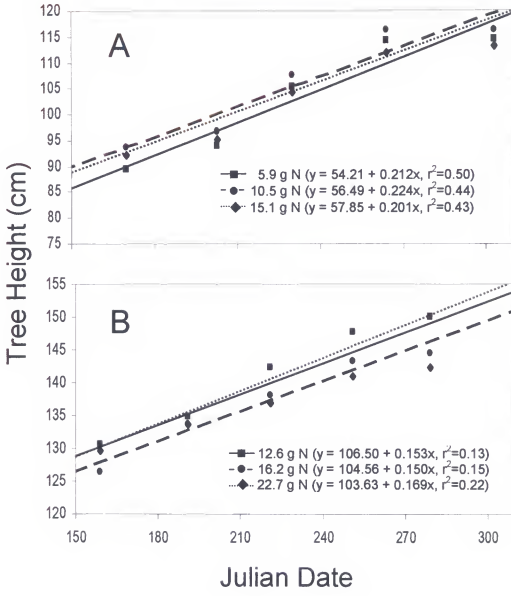

Figure 3.4. N application rate and scheduling effects on young 'Hamlin' average tree height at the Horticultural Research Unit in Gainesville, FL in 1994 (A) and 1995 (B) based on fertilization rate. Analysis of covariance was completed for each date shown. Slopes of individual regression lines were not significantly different in 1994 or 1995. Adjusted LS means are shown, n = 33.

on analysis of covariance fertilizer application frequency had a significant effect on plant height on a few dates. However, regression analysis and T-tests showed that slopes of lines based on N application frequency were not significantly different (Fig. 3.4. B).

Percent N concentration in the leaves was not significantly different related to irrigation scheduling or N application frequency effects. Mean N concentration among all treatments was 3.31 % in 1994 and 2.47 % in 1995.

Following 2 years of different irrigation scheduling and N application timing all trees were visually rated based on overall size and appearance in Sept, 1995. Irrigation scheduling had a slight effect ($p \leq 0.0702$). Trees subject to the 20-1 SWD irrigation schedule had a significantly higher mean visual rating than 20 SWD treated trees (3.2 vs. 2.8) (Fig. 3.5.). Trees under the 20-2 SWD regime were not visually different than 20 SWD treated trees or 20-1 SWD treated trees.

Interestingly, different irrigation regimes based on presence or absence of growth flushes had some significant effects on growth activity and overall visual appearance, but not growth. Marler and Davies (1990) found that irrigation scheduling based on 65% SWD significantly delayed all growth flushes except the spring flush. They also found that irrigation scheduling based on 45% SWD significantly delayed the initiation of the third growth flush. Zekri and Parsons (1988) also showed that no irrigation or drip irrigation which did not reach much of the root zone reduced growth during the summer flush of grapefruit trees. The observation that less irrigation during the second flush of 1994 reduced growth

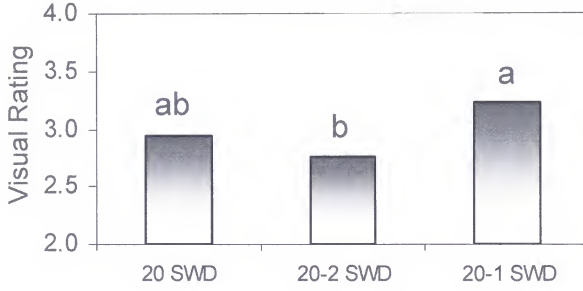

Figure 3.5. Effects of irrigation scheduling on the visual rating of young 'Hamlin' orange trees. Ratings were based on observations by two different observers on 8 Sept 1995 and 15 Sept 95 following nearly 2 years of different irrigation scheduling treatments. Scale ranged from 1 to 5, 1 representing poor growth and overall appearance and 5 representing excellent growth and overall appearance. Different letters represent significantly different means based on Duncan's multiple range test ($p \leq 0.05$), n=33. Means with the same letter are not significantly different.

along with these observations suggests that during a period without regular rainfall (Appendix A) 45% SWD may be too severe for optimum growth. In contrast, Marler (1988) observed that growth as defined by shoot and leaf expansion rates as well as ultimate size, was little affected by irrigation treatment. This was supported by visual comparison of treatments in this study. Trees with the best overall appearance got less total water for 2 years of the study (Fig. 3.5). However, differences in visual ratings were small and may not have practical significance.

Regardless of irrigation scheduling, trunk diameters and plant heights were similar throughout the growing season (Fig. 3.3 + 3.4). In addition, the general number and timing of most flushes observed was similar to those reported in other studies (Marler and Davies, 1989; 1990) with the exception of a 4th low level period of growth activity observed in 1995 (Fig. 3.2.B). However, additional late season flushes are reported in the literature (Maurer and Davies, 1993). These data suggest that the growth activity of 'Hamlin' trees in this study was, in general, similar to previous reports and lack of irrigation scheduling effects was not due to any aberrant or unusual growth patterns.

Fertilization frequency had no effect on young 'Hamlin' orange tree growth. Unless leaf N concentrations are toxic or deficient, young citrus trees are usually unresponsive to different application timings (for review see Davies, 1997)). Guazzelli et al., (1996) examined leaf N levels of young citrus trees from the nursery and found that initial levels of leaf N from 1.7 to 4.1 % had no significant effect on growth during the first year in the field. This observation was

more striking when one considers that 0 to 0.75 kg N•tree^{-1}•year^{-1} was applied to the trees during that same year. Willis et al. (1990) found that fertilization frequency usually did not affect citrus tree growth, except for trees on 'Carrizo' citrange rootstock. Young citrus trees from the nursery likely have large stores of N in branches and stems in addition to the leaves which may be remobilized for growth (Legaz et al., 1995) this may account the lack of fertilization frequency effects in studies using young trees. Finally, observed increases in trunk diameter between the first and second year are similar to gains observed in young 'Redblush' grapefruit trees on 'Swingle' citrumelo rootstock (Maurer and Davies, 1993). These reports again suggest, as with irrigation scheduling, that lack of fertilization frequency effect was real and the not the product of unusual tree growth patterns.

Freeze hardiness development. From Nov 1994 until Mar 1995 freeze hardiness was determined weekly at three different temperatures –3, -6 and –9° C (Fig. 3.6). Irrigation scheduling had a significant effect on freeze hardiness of the 'Hamlin' orange leaves at –3, -6, and –9 °C ($p \leq 0.0001$, $p \leq 0.0001$, and $p \leq 0.005$ respectively). With the exception of the 3rd freeze test (Julian day 346) trees irrigated under the 20-1 SWD treatment had significantly less EL and were more freeze hardy than 20 SWD trees at –3° C for the first five tests of the study (Fig. 3.6.A). On the 4th and 5th test dates 20-2 SWD and 20-1 SWD treated trees were not significantly different. From Jan to Mar of 1995 irrigation scheduling had no significant effect

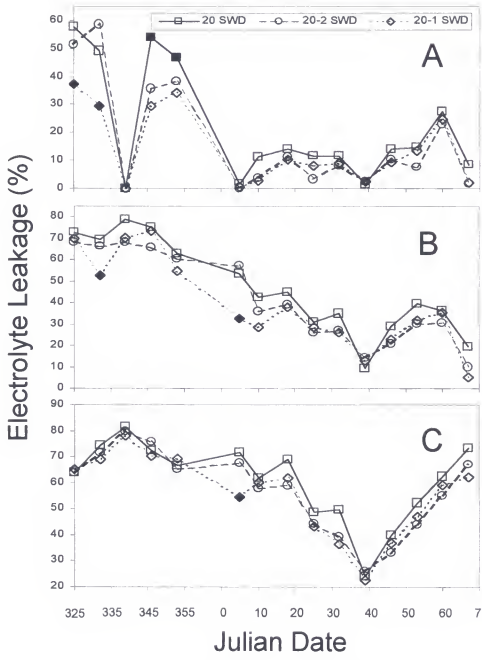

Figure 3.6. Effects of irrigation scheduling on the injury of young 'Hamlin' orange leaves at -3 (A), -6 (B), and -9 °C (C) during the winter 94-95 based on irrigation scheduling. Three leaf discs from 18 randomly sampled leaves from each treatment were tested at each of the 3 test temperatures. Filled markers represent significant mean separation based on Duncan's multiple range test, $p \leq 0.05$, n=18.

on leaf hardiness at –3° C for any individual date, however overall EL was reduced to an average of about 10% instead of the 30 to 50% observed prior to those dates. Irrigation scheduling had a significant effect on EL at –6° C on Julian dates 332 and 5. On both dates 20-1 SWD treated trees displayed significantly less leakage from leaf discs than either scheduling treatments 20-2 SWD or 20-1 SWD. Overall EL decreased until Julian date 38 to 13 % then increased again to 33% before returning to 12 % on Julian date 67. Trees with 20-1 SWD scheduled irrigation had significantly less EL at –9°C than the 20 SWD and 20-2 SWD treated trees only on Julian day 5. Overall EL values of leaf discs at –9 °C decreased to 24% until Julian day 38 and then increased until the end of the study.

In 1995 freeze tests were only completed once every 30 days (Fig. 3.7). Freezing tests were terminated following pump failure during a –9 °C freeze on day 358, after which trees were > 95% defoliated leaving no leaf material for freeze tests. Although irrigation played a significant effect in the overall model based on the large sample size, there were no significant differences among irrigation scheduling treatments on any individual date.

Electrolyte leakage is an indirect measure of leaf freeze hardiness. Other methods include using EL over a range of temperatures to determine leaf killing point (LKP), LT_{50}, and vital stains. The most accurate method for determining plant hardiness for containerized seedlings is to observe plant regrowth after freezing *in vivo* (Yelenosky, 1985). However, in the field the EL technique is the only practical way to examine changes in freeze hardiness over time.

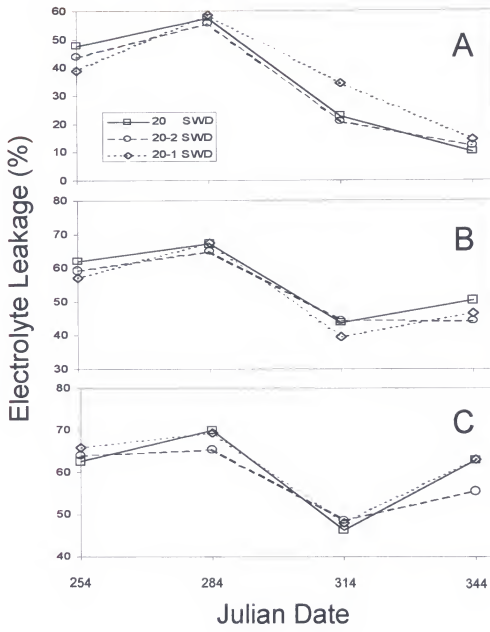

Figure 3.7. Effects of irrigation scheduling on freeze injury of young 'Hamlin' orange leaves at -3 (A), -6 (B), and -9 °C (C) in 1995 based on irrigation scheduling. Three leaf discs from 30 randomly sampled leaves from each treatment were tested at each of the three test temperatures. Filled markers represent mean separation by Duncan's multiple range test, $p \leq 0.05$, n=30.

Electrolyte leakage is considered a general measure of membrane damage. To address discrepancies that might occur between regrowth studies and EL determination of freeze hardiness, a controlled freeze was conducted with 2-year-old greenhouse grown 'Hamlin' orange on 'Swingle' citrumelo rootstock (Appendix B). Acclimating 'Hamlin' trees at 6 weeks under 21 °C days and 10° C nights resulted in about 50% EL at –6° C. In comparison, freeze-acclimated intact trees frozen to –6° C showed nearly 100% leaf drop after 3 days, but had no stem damage. These data were comparable to Yelenosky's (1990) research on the freeze hardiness of 'Hamlin' trees by regrowth tests. He found no stem dieback and that leaves were least damaged at –6.7 °C, but used a lower acclimating temperature regimen. Electrolyte leakage data for each temperature was used directly in our analysis due to the inherent error in fitting curves to freezing data to find inflection points. Few data points and choice in best fit analysis are the major sources of this error.

Decreases in irrigation amounts during the growing season resulted in decreased EL when leaf discs were exposed to –3 and –6 °C freezes. The greatest EL reduction was about 20% and occurred early in the season from Julian day 325 (1994) to Julian day 5 (Fig. 3.6.A+B). This is interesting since irrigation schedule 20-2 SWD had no effect on tree height and a only resulted in a small significant reduction in trunk diameter. However, to take advantage of this potential increase in freeze hardiness, the growing season must have sufficient periods with low or no rainfall which is unusual in Florida. Irrigation scheduling that reduces total water applied per season, but does not result in a

reduction in growth as determined by trunk diameter and plant height can increase freeze hardiness during dry years. This trend was observed by Davies et al., (1981) for 'Orlando' tangelo trees. However, Young (1970), Cooper (1963), and Yelenosky (1975; 1978) observed that exposure to low temperature regimes reduced freeze injury to citrus trees and low temperature was the major stimulus for freeze acclimation. In the winter of 1995 no significant differences were seen based on irrigation scheduling on individual dates, but this was likely due to the large time interval between freezing tests.

Overall trends in freeze data suggest differently regulated mechanisms of freeze acclimation. Freeze hardiness at –3 °C (Fig. 3.6.A) changed rapidly on a weekly basis probably based on changes in air temperature, which ultimately affected plant microclimate. Such major rapid changes were not found in % EL at –6 ° or –9 °C (Fig. 3.6.B+C). In addition, from Julian day 38 to 60 EL at –6° C peaked and then dropped off, typical of a short deacclimation-acclimation process, while overall EL at –9° C steadily increased suggesting steady deacclimation.

Alternatively, acclimation could occur in one linear set of processes. Trees in this study showed rapid acclimation and deacclimation based on EL at –3 ° throughout the winter (Fig. 3.6). However at –6 and –9 ° C acclimation and deacclimation occurred without as much fluctuation (Fig 3.6). Electrolyte leakage values at –6 and –9° C showed a gradual decline (increase in freeze hardiness) and then at –9° C steadily increased (decrease in freeze hardiness) in the winter of 1994-95. Electrolyte leakage at –6 °C increased and then decreased during

the same period. These data suggest multiple mechanisms might be involved in the induction of freeze hardiness following exposure to various environmental stimuli.

Freeze recovery study. Following the freeze on day 358 in 1995 we conducted a freeze recovery study using different N application rates. Based on the analysis of covariance there was no difference in either scion or rootstock trunk diameter (Fig. 3.8.A+B). Both scion and rootstock trunk diameters increased about 10% from Julian date 151 to 230 in 1996. This 10% increase in trunk diameters regardless of N application rate are in agreement with previous studies on mature non-bearing 'Hamlin' trees where fertilization resulted in no significant change in tree growth following a freeze (Davies and Maurer, 1990). However, this was expected since even young citrus trees have a reasonable N storage capacity in branches and trunks.

In theory, irrigation scheduling practices could be altered to provide additional freeze hardiness and thus some increased protection during early freezes. This research suggests that acclimation rates could also be increased in the same manner. Such practices as irrigating at 20% SWD through the first flush and at 45% during the remainder of the year may even slightly improve growth and overall appearance. Large differences in fertilization rates following a severe freeze had no effect on growth of young non-bearing 'Hamlin' trees.

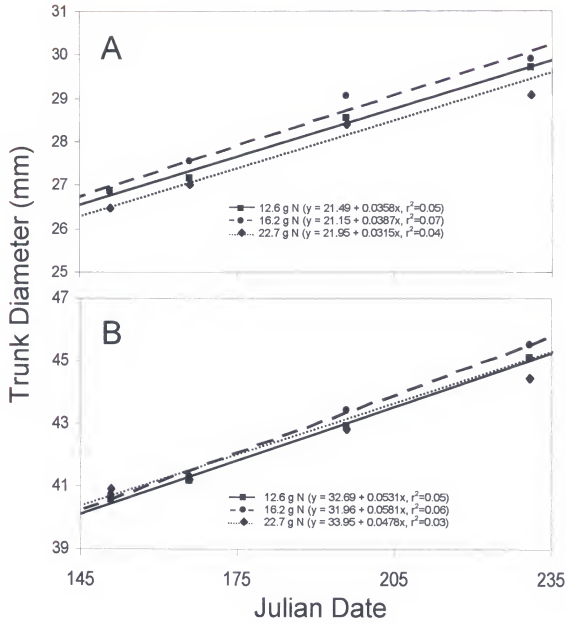

Figure 3.8. Fertilization application rate effects on trunk diameter of young 'Hamlin' orange trees (A) budded on 'Swingle' citrumelo (B) rootstock at the Horticultural Research Unit in Gainesville, FL in 1996. Slopes of regression lines shown were not significantly different in 1994 or 1995. Adjusted LSmeans shown, n=30.

Interestingly, the freeze hardiness data from 1994 suggest a complex mechanism of freeze acclimation and deacclimation. Changes in acclimation and deacclimation simultaneously of young 'Hamlin' orange trees at –3, -6, and -9 °C suggest that acclimation and deacclimation rates can change at different levels of freeze hardiness independent of one another.

CHAPTER IV
FREEZING TOLERANCE AND GROWTH CHARACTERISTICS OF USDA INTERGENERIC CITRUS HYBRIDS 'US 119' AND SELECTION 17-11

Introduction

The Florida citrus industry lost over 1 billion dollars in tree and crop damage in the 1980s alone due to freezes. In some of these freezes tree and crop losses were reduced by using freeze protection methods such as petroleum heaters, soil banking, tree wraps and microsprinkler irrigation (Davies et al., 1984; Jackson et al., 1983; Parsons et al., 1982). All of these methods are successful in preventing some damage to young citrus trees during a freeze, especially microsprinkler irrigation in conjunction with tree wraps (Rieger et al.,1985; 1986), but water availability and cost may limit its use in the future.

Another avenue for decreasing citrus tree loss due to freezes is breeding for more hardy citrus. Improved freeze-hardiness through breeding citrus could provide long term reliable and economic freeze protection (Yelenosky, 1985). The goal of such a program is not necessarily to move citrus planting northward in the United States, but rather to provide hardier trees for current growing locations (Yelenosky, 1985).

A USDA breeding program in Orlando, FL has been in the process of developing more freeze-hardy citrus scions and rootstocks for decades (Barrett, 1982). *Poncirus trifoliata* (L.) Raf. in particular has been used to introduce freeze hardiness and winter dormancy traits into breeding lines (Barrett, 1990; 1994). 'US 119' and selection 17-11 are both products of this breeding program. 'US 119' was the result of an intergeneric cross of a selection of (*C. paradisi* Mac. c.v. Duncan X *P. trifoliata* (L.) Raf) X *C. sinensis* Osb. c.v. Succory (Fig. 4.1.A). It was released primarily due to its resistance to CTV (Garnsey et al., 1981; 1987) and lack of unpalatable fruit qualities often associated with hybrids having *P. trifoliata* as a parent (Barrett, 1990). There was also anecdotal evidence that 'US 119' stems were hardy to –12.2 °C (Barrett, 1990), suggesting it was substantially more freeze hardy than any commercial citrus cultivar in the U.S.

USDA selection 17-11 was chosen from progeny produced following the cross of *C. grandis* c.v. North East by (*C. paradisi* Mac. c.v. Duncan X *P. trifoliata* (L.) Raf) (Fig. 4.1.B) (Wayne Sherman, personal communication). Nothing has been reported about the freeze hardiness of 17-11.

The primary objective of this study was to determine the freeze hardiness of both intergeneric hybrids and compare it to commercial cultivars such as 'Hamlin', the most widely planted sweet orange cultivar in Florida, and satsuma mandarin, the hardiest commercial cultivar used in the world. Growth characteristics of each hybrid and their relationship with freeze hardiness were also examined.

[*Citrus paradisi* Mac. c.v. Duncan X *P. trifoliata* (L.) Raf]
X *C. sinensis* Osb. c.v. Succory

C. grandis N.E. X [*C. paradisi* Mac. cv. Duncan X
P. trifoliata (L.) Raf]

Figure 4.1. Parentage and leaf morphology of 'US 119' (A) and selection 17-11 (B).

Materials and Methods

Plant material and cultural practices. Budwood collected from existing stock plants of 'US 119' and selection 17-11(provided by H. Barrett, USDA, Orlando, FL) and satsuma mandarin was budded grafted onto commercially propagated 'Swingle' citrumelo liners. Rootstock shoots were cut following ample growth of the bud in the greenhouse. Following 3 weeks of protected growth in the greenhouse, 10 individuals of each scion-rootstock combination were planted at the Fifield Horticultural Research farm in Gainesville, FL in May of 1993. On 3 May 1995, 10 additional trees of each scion-rootstock combination were planted at the University of Florida Horticultural Research Unit in Gainesville, FL.

Fertilizer (8N-2P_2O_5-8$K_2$0-2Mg, Seminole Fertilizer) was applied about every 5 weeks (for a total of 6 times) at 0.10, 0.14, 0.22, and 0.33 kg N/tree/year in 1993, 1994, 1995, and 1996 respectively, based on recommendations for young citrus trees by Koo et al. (1984). Trees were irrigated when soil water depletion exceeded 30% (Marler and Davies, 1990) as determined by a Troxler model 4300 neutron probe (Research Triangle Park, N.C.). Weeds were controlled using glyphosate as needed. Neither trunk wraps or microsprinkler irrigation were used for freeze protection.

Plant growth measurements. Procedures were essentially the same as in Chapter 3 (page 36) and are briefly described here. All trees were examined

weekly to determine the presence or absence of a growth flush. For the tree to be scored as flushing there had to be rapidly elongating vegetative buds or expanding leaves present on at least three different branches, thus avoiding isolated bud-break occurring occasionally on individual shoots throughout the growing season. The growth was considered finished when all leaves from the current flush were fully expanded.

Tree height was measured monthly from the soil to the highest apical meristem. Scion and rootstock trunk diameters were measured at the graft interface, which was typically 20-25 cm above the soil line, using a caliper oriented north-south. Scion and rootstock measurement locations were marked with permanent ink to insure repeatability of trunk diameter data. Tree height and scion and rootstock trunk diameters were recorded monthly.

Leaf N Analysis. Leaf N analysis was conducted in the same manner as described in Chapter 3 (pages 39 to 40). Briefly, 10 fully expanded leaves from the spring flush, free of obvious damage or infestation, were sampled in late summer from each scion-rootstock combination. Leaves were washed in distilled water and placed in a drying oven at 70 °C for 72 h. Tissue was then ground and stored in scintillation vials until a 250 mg sub-sample from each vial was placed in a Pyrex digestion tube. Total N was then determined using the micro Kjeldahl procedure (Wolf, 1982). Concentrated H_2SO_4 (2.5 ml) was added and samples digested for 24 h followed by repeated rounds of neutralizing with H_20_2 and heating on a 375 °C digestion block until samples were clear. Samples were

cooled and volume brought up to 25 ml with distilled water. The solution was then passed through filter paper and stored at 4°C in scintillation vials until samples were analyzed by the University of Florida Analytical Research Lab in Gainesville, FL.

Freeze hardiness determination. Freeze hardiness of scion-rootstock combinations was determined using the leaf electrolyte leakage (EL) method as described in the materials and methods section of Chapter 3 (pages 38 to 39) with the following additional notes. Intergeneric hybrids 'US 119' and selection 17-11 show variable leaf morphology (Fig. 4.1). Unifoliate, bifoliate, and trifoliate leaves are present on both selections, but unifoliate leaves predominate. Leaves were selected randomly for the disc freezing procedure, however, discs were sampled from only the large center leaflet of any bifoliate or trifoliate leaves to keep morphology consistent in the leaf disc samples.

Statistical analysis. This completely randomized design was analyzed using a general linear model procedure (GLM) to perform an analysis of variance (ANOVA). For growth flush data ANOVA tables were generated for each date and significant difference in means determined via Duncan's multiple range test (DMRT). Trunk diameters and plant heights were evaluated using an analysis of covariance to standardize any initial differences and generate adjusted least squared means. Regression analysis was also performed on hybrid trunk

diameter and tree height data. All statistical calculations were completed using Statistical Analysis Software (SAS Institute, Cary, NC).

Results and Discussion

Growth and Development. 'US 119' had four distinct growth flushes in 1994 (Fig. 4.2.A). USDA selection 17-11 also had four growth flushes, however, a higher % of trees continued growing than for 'US 119'. During the second flush of 1994 'US 119' was flushing significantly more than selection 17-11 for 3 weeks, although growth began about 2 weeks behind the second growth flush for selection 17-11. 'US 119' flushed almost exactly one week behind 17-11 during the 3rd growth flush. The percentage of 17-11 trees actively growing was significantly greater during the beginning of its third growth flush than that for 'US 119'. During the 2 weeks prior to the end of its third growth flush 'US 119' showed more growth flush activity than selection 17-11. In addition, selection 17-11 had a significantly reduced final flush for Julian dates 285-330.

The only significant difference in growth flush patterns came during the first flush for 'US 119' during Julian dates 105,120, and 142 in 1995. 'US 119' was flushing more than selection 17-11 (Fig. 4.2.B). Otherwise both selections had three distinct growth flushes followed by a fourth period of low-level flushing activity from Julian date 235 to 275. Each flush in 1995 occurred 30 to 60 days

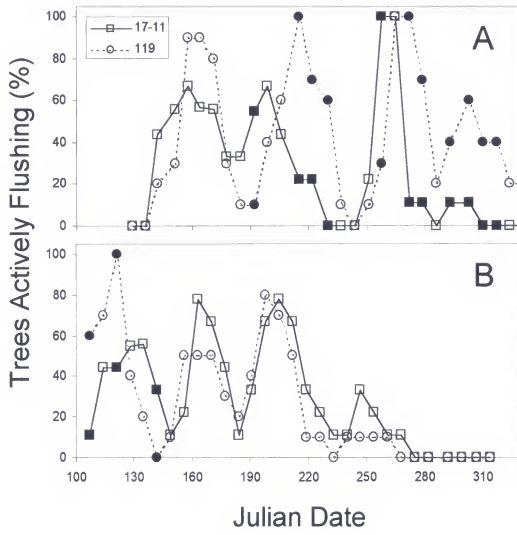

Figure 4.2. Differences in USDA selection 17-11 and 'US 119' growth flush patterns in 1994 (A) and 1995 (B). at the Horticultural Research Unit in Gainesville, FL. Tree growth activity was examined weekly during the growing season. Filled markers represent significantly different means based on Duncan's multiple range test, $p \leq 0.05$, n=10.

earlier than corresponding flushes in 1994. In general, the magnitude of growth flushes for 'US 119' was 20% lower (in terms of % trees actively growing) in 1995 than in 1994 for flushes 2, 3, and 4.

There were no significant differences in trunk diameter between selection 17-11 and 'US 119' at any date in 1994 (Fig. 4.3.A) or 1995 (Fig. 4.3.B) based on analysis of covariance and T-tests on LSmeans. Additionally, slopes for the regression lines produced for selection 17-11 and 'US 119' were similar in 1994 and 1995. Similarly, trunk diameters of 'Swingle' citrumelo rootstock were not significantly different at any date examined in 1994 (Fig. 4.4.A) and 1995 (Fig. 4.4.B).

Tree height for 'US 119' and selection 17-11 was similar at all dates measured in 1994 and 1995 based on analysis of covariance and t-tests on adjusted LS means (Fig. 4.5.A + B).

Leaf N concentration was not significantly different between 'US 119 and selection 17-11 in 1994 or 1995 (data not shown). Similar results were obtained in 1995. In 1994 and 1995 mean % N for all intergeneric citrus scion hybrids tested was 4.0 and 2.7, respectively.

In summary, 'US 119' and selection 17-11 grow in a similar manner as determined by scion and rootstock trunk diameter and tree height. Selection

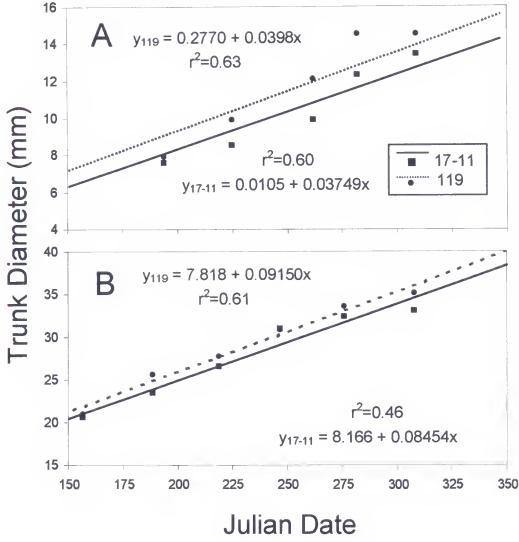

Fig. 4.3. Differences in the trunk diameters of USDA selection 17-11 and 'US 119' in 1994 (A) and 1995 (B) in a block at the Horticultural Research Unit in Gainesville, FL. There was no significant difference in plant height between 119 and 17-11 at any date. Adjusted least square means are shown. Slopes of regression lines were not significantly different in 1994 or 1995, n=10.

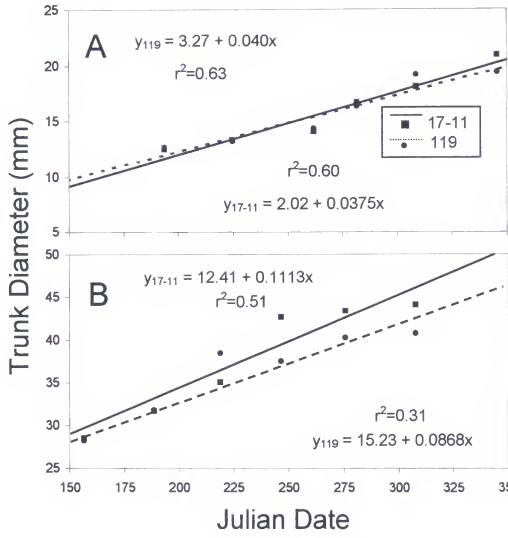

Fig. 4.4. Effects of USDA selection 17-11 and 'US 119' on trunk diameter of 'Swingle' citrumelo rootstock in 1994 (A) and 1995 (B) in a block at the Horticultural Research Unit in Gainesville, FL. There was no significant difference in plant height between 'US 119 and 17-11 at any date. Adjusted least square means are shown. Regression lines are for adjusted means, slopes of lines were not significantly different in 1994 or 1995, n=10.

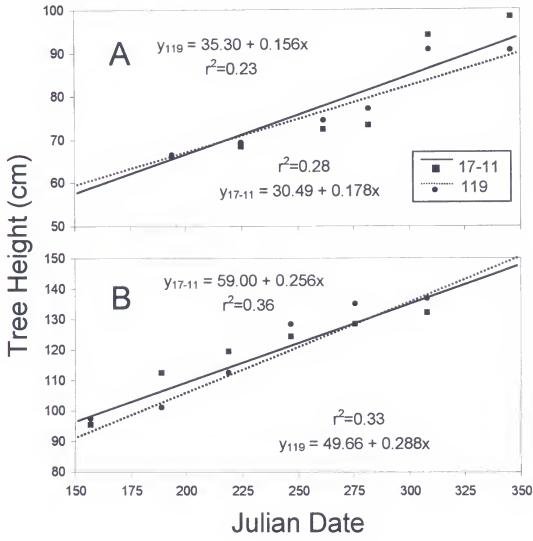

Fig. 4.5. Differences in plant height for USDA selection 17-11 and 'US 119' in 1994 (A) and 1995 (B) in a block at the Horticultural Research Unit in Gainesville, FL Adjusted least square means are shown. Regression lines are for adjusted mean squares, slopes of lines were not significantly different in 1994 or 1995, n=10.

17-11 tends to be more upright morphology and sparsely branching with numerous large thorns. Selection 17-11 was also observed to be extremely susceptible to rust mite damage during the 4 years of this study. In comparison, 'US 119' was slightly more compact with fewer thorns and was more densely foliated. General visual observations for 'US 119' were in agreement to those of Barrett (1990) who described 'US 119' as "a moderately vigorous grower, with dense, dark green foliage."

'US 119' flushes later than selection 17-11 and this growth activity may continue into early winter (Fig. 4.2.A) in a relatively dry warmer year, such as 1994 as compared to 1995 (Appendix A). This may increase its susceptibility to early freezes during such a year, especially for new shoots and young leaves in comparison to selection 17-11. Growth flush patterns of selection 17-11 were similar to those of 'Hamlin' orange in 1994 (Fig. 3.2.A), but selection 17-11 stopped flushing sooner. This suggests that 17-11 began quiescence and possibly freeze acclimation sooner than either 'Hamlin' orange or 'US 119', in this relatively dry year (Fig. A.2). Growth cessation, especially bud quiescence does not increase citrus freeze hardiness per se (Young, 1970), but lack of growth activity later in the year is generally associated with increases in freeze hardiness (Cooper et al., 1963). Both 'US 119' and 17-11 stopped growing actively about 2 weeks sooner than 'Hamlin' orange in 1995 (Fig. 4.2.B and Fig. 3.2.B), suggesting that in this particular year both were entering a 'nonapparent' growth period earlier than 'Hamlin' orange.

Freeze Acclimation and Hardiness Development. Leaf freeze hardiness of 'US 119, selection 17-11, and satsuma mandarin was examined monthly in 1993 to determine if either of the intergeneric hybrids was more hardy than satsuma mandarin, which is the hardiest commercially produced citrus species (Yelenosky, 1985). No significant differences in leaf disc EL were found at –4, -8, or –12 °C at any of the dates tested for selection 17-11, 'US 119', or satsuma mandarin. By Julian date 294 all selections were hardy to –4 °C showing negligible damage as determined by EL (Fig. 4.6.A). EL decreased to less than 25% for all selections by Julian date 361 (Fig. 4.6.B).

Samples for freeze analysis were taken weekly starting in Nov 1994 through Mar 1995 to determine if there were more frequent fluctuations in leaf freeze hardiness. 'Hamlin' leaves were compared to selection 17-11 and 'US 119 during this series of freeze tests. 'US 119' was already significantly more hardy than either selection 17-11 or 'Hamlin' orange during early acclimation from Julian dates 325 to 353 (Fig.4.7.A). Both selection 17-11 and 'US 119' had significantly less EL at –3°C than 'Hamlin' orange from Julian dates 5 to 24, but neither hybrid was significantly different from one another (Fig. 4.7.A). 'Hamlin' again had significantly more EL than either hybrid during Julian dates 52 to 66 (Fig. 4.7.A). Both selection 17-11 and 'US 119' exposed to –6 °C showed significantly less EL from Julian date 325 (1994) to 52 (1995) (Fig. 4.7.B) than 'Hamlin' orange leaf discs. Electrolyte leakage between selection 17-11 and 'US 119' was not significantly different except at Julian dates 339, 446, and 353 when 17-11 had between a 100 and 66% reduction in EL.

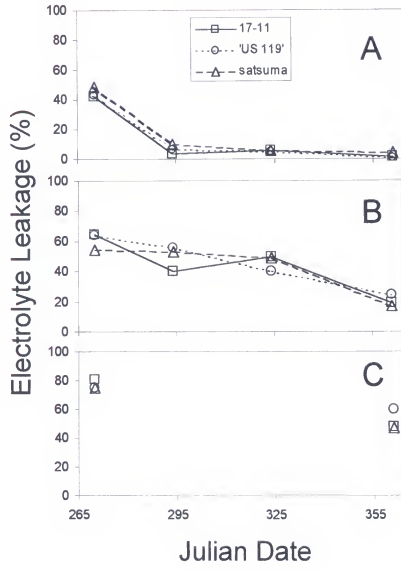

Figure 4.6. Evaluation of freeze acclimation and hardiness of USDA selection 17-11, 'US 119', and satsuma mandarin located at the Fifield Horticultural Research Farm in Gainesville, FL in 1993 at -4 (A), -8 (B), and -12 °C (C). No significant differences among cultivars were observed, n=8.

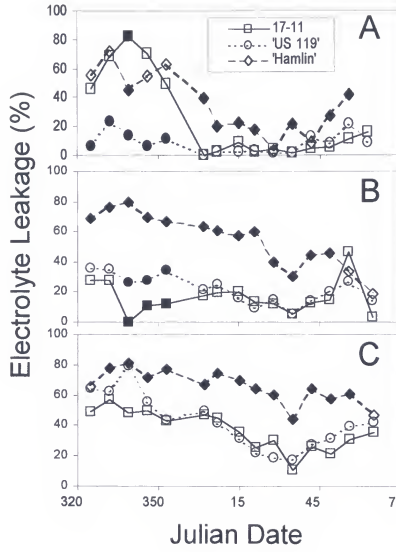

Figure 4.7. Evaluation of freeze acclimation and hardiness of USDA selection 17-11, 'US 119', and 'Hamlin' orange in 1994 and 1995 at -3 (A), -6 (B), and -9 °C (C). Trees were located at the Horticultural Research Unit in Gainesville, FL. Analysis of variance was completed for each date shown. Solid markers represent significantly different means based on Duncan's multiple range test, $p \leq 0.05$, n=10.

compared to 'US 119' (Fig. 4.7.B). 'US 119', selection 17-11, and 'Hamlin' orange had similar amounts of EL at –6 °C on Julian date 59 and 62. With the exception of 3 dates (Julian dates 325, 346, and 66) selection 17-11 and 'US 119' had significantly less EL than 'Hamlin' orange leaf discs frozen to –9 °C.

In 1995 'Hamlin' orange and satsuma mandarin were compared simultaneously with selection 17-11 and 'US 119'. As expected at –3 °C on Julian date 254, EL was significantly less for 'US 119', selection 17-11, and satsuma mandarin than for 'Hamlin' sweet orange (Fig. 4.8.A). 'US 119' had significantly less EL than either selection 17-11 or satsuma mandarin on this date. A month later EL was significantly different among cultivars (Julian date 284). In order of least to greatest EL (therefore in order of decreasing freezing hardiness) were selection 17-11, 'US 119', satsuma mandarin, and 'Hamlin' orange. On Julian date 314 and 344 selection 17-11, 'US 119', and satsuma mandarin all had significantly less EL than 'Hamlin' orange (Fig.4.8.A). Leaves tested fell into three different groups for EL measured at –6 °C (Fig. 4.8.B). Selection 17-11 had significantly less EL than satsuma mandarin and 'US 119', all of which had significantly less EL than 'Hamlin' orange (Fig.4.8.B). Differences in EL between selection 17-11, 'US 119', and satsuma were small. There were no significant differences in EL on day 254 and 284 at –9 °C (Fig. 4.8.C). However, on Julian date 314 and 344 groupings were the same as those at –6 °C (Fig. 4.8.C). In short, 'Hamlin' orange showed significantly more EL at –9 °C than 'US 119' and satsuma mandarin, which had similar EL levels.

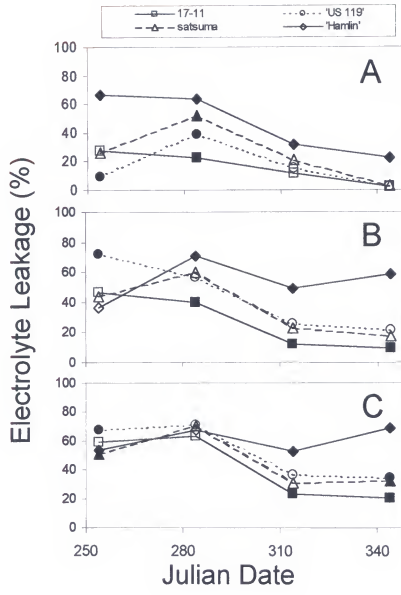

Figure 4.8. Evaluation of freeze acclimation and hardiness of USDA selection 17-11 and 'US 119' satsuma mandarin, and 'Hamlin' orange in 1995 at -3 (A), -6 (B), and -9 °C (C). Trees were located at the Horticultural Research Unit in Gainesville, FL. Solid markers represent significantly different means based on Duncan's multiple range test, $p \leq 0.05$, n=6.

Selection 17-11 showed significantly less EL than the other citrus selections at both of these dates.

All four citrus selections were tested every 3 weeks, from Sept 1996 until Mar 1997, to examine hardiness trends for a fourth year. At –3 °C there were no significant differences among selections in EL (Fig. 4.9.A). At –6° C 'Hamlin' had significantly more EL for Julian dates 306, 324, 355, and 48. For Julian dates 306, 324, 355, and 48, 'US 119', selection 17-11, and satsuma mandarin had less EL than 'Hamlin' orange, but were not significantly different from each other (Fig. 4.9.B), except for Julian date 66 where 'Hamlin' and 'US 119' had similar EL levels following exposure to –6 °C (Fig. 4.9.B). Large significant differences arose in EL on Julian date 355 (1996) and 4 (1997) when 'US 119', selection 1711, and satsuma mandarin leaves had significantly less EL than 'Hamlin' orange following exposure to –9 °C (Fig. 4.9.C). In addition, for dates 25 and 48 selection 17-11 had significantly less EL than 'US 119', satsuma, and 'Hamlin'.

Electrolyte leakage is an indirect measure of freeze hardiness (Flint et al., 1967). Leaf freeze hardiness of two intergeneric citrus hybrids and two citrus species was evaluated for 4 years. There were several trends that became clear, although data varies by year and Julian date. Selection 17-11 is generally most hardy followed by 'US 119', satsuma mandarin, and 'Hamlin' orange. However, in some years there was little significant difference in leaf EL among selection 17-11,'US 119', and satsuma (Fig. 4.6.). This was likely the result of too few sampling dates. When significant differences were observed,

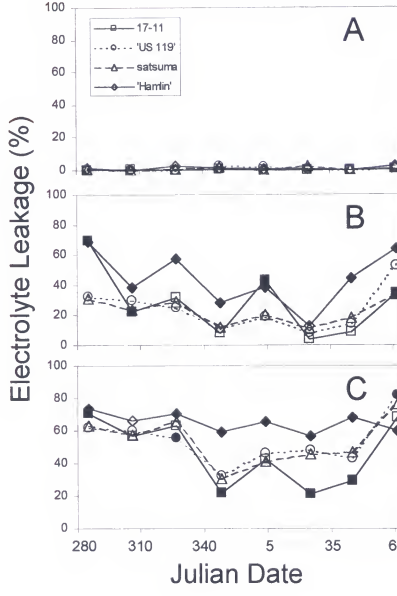

Figure 4.9. Evaluation of freeze hardiness and acclimation of USDA selection 17-11, 'US 119', satsuma mandarin, and 'Hamlin' orange in 1996 and 1997 at -3 (A), -6 (B), and -9 °C (C). Leaves were sampled from trees planted at Fifield Horticultural Research Farm in Gainesville, FL. Solid markers represent significantly different based on Duncan's multiple range test, $p \leq 0.05$, $n = 10$.

selection 17-11 was hardier than 'US119' and satsuma mandarin at least once during the time period tested.

'Hamlin' orange acclimated later and deacclimated faster than the other selections in years where EL data was sampled more often. Selection 17-11 seemed to more closely follow the same general acclimation pattern as 'Hamlin' (Fig. 4.7.A, Fig. 4.9.B), although 1994 EL data for 17-11 was problematic at –3 °C. Leaf EL values were consistently higher for the first 3 freeze tests at –3 °C than values for leaf samples at –6 °C. On rare occasions singular aberrant values appear for trees being tested but these values for selection 17-11 for 3 consecutive weeks are unexplained and were not observed during any other time period during 4 years of freeze tests.

Finally, in the winter of 1994-95 and 1996-97 'Hamlin' EL averaged 23 and 18%, respectively, when leaf discs were exposed to –6 °C. Based on our comparison of acclimated vs. non-acclimated 'Hamlin' on 'Swingle' citrumelo trees (Appendix B), whole trees with these EL values would have experienced no stem damage and little or no leaf damage. This is in good agreement with Yelenosky's (1990) data that showed the 1-year-old 'Hamlin' orange trees on rough lemon rootstock (*C. jambhiri* Lush.) exposed to 15.6 °C days and 4.4 °C nights for 6 weeks resulted in no leaf loss or stem injury following 4 consecutive hours of –6.7 °C. These results help to establish that this technique has merit in determining freeze hardiness and acclimation rates over time. 'US 119' also has survived freezes of –12.2 C (air temperature) in 1981 (Barrett, 1990). The trees were completely defoliated at this temperature, but bloomed and set fruit during

the following year. We observed similar defoliation in both US 119 and 17-11 following two freezes in 1996 when air temperature reached –10 °C. In addition, defoliation occurred to a lesser extent when air temperatures reached –9 °C in 1995. In both years defoliation occurred via abscission at the petiole-leaf interface with the petiole remaining on the tree until spring flush occurred. This is typically a sign of less severe freezing injury than dead leaves persisting on a tree following a severe freeze (Davies, personal communication).

In summary, 'US 119' appears to be hardier to freezes than all commercially produced citrus cultivars in the United States, but is not hardier than satsuma mandarin. 'US 119' should be a useful source for parent selection for breeding freeze-hardy commercial citrus scion cultivars, especially with its apparent resistance to CTV. Selection 17-11, in addition to being hardier than sweet orange may be potentially hardier than 'US 119' and satsuma mandarin and therefore is also a potential candidate parent for citrus breeding programs. In addition, 'US 119' and 17-11 show different patterns of growth activity and different levels of freeze hardiness on the same dates suggesting different responses to temperature, although subsequent experiments are needed to establish this.

CHAPTER V
RAPID FREEZE ACCLIMATION OF *PONCIRUS TRIFOLIATA* SEEDLINGS EXPOSED TO 10 °C AND LONG DAYS

Introduction

Citrus is a freeze-tender evergreen of tropical and subtropical origin capable of freeze acclimating (Yelenosky, 1985). However, even the hardiest citrus species do not approach the freeze tolerance level of many woody temperate species, some of which can survive freezing in liquid nitrogen when acclimated (George et al., 1974). When fully acclimated the minimum leaf temperature at which *C. sinensis* can survive is about -6.7 °C (Yelenosky, 1985).

Long-term freeze acclimation of citrus has been studied for many years. Young (1970) used 9 to 12-month-old seedlings of many different cultivars and examined freeze acclimation based on diurnal temperature regimes of 21/10, 16/4, 10/-1, and 7/-3.3 °C. Seedlings were exposed to each temperature regime for 2 weeks successively and degree of acclimation was determined by freezing leaves and then examining the level of watersoaking. His rankings of citrus species in general correlated with field observations following an actual freeze (Cooper, 1965). Citrus trees freeze-acclimated at low temperatures under short (8 h) days were more hardy than those exposed to the same temperatures under long (16 h) days (Young, 1961). Yelenosky (1978) found that the leaves of

'Valencia' oranges on 'Rusk' citrange rootstock would eventually freeze harden to -6.7 °C after 28 days of exposure to 10 °C. These experimentally determined values agree with the observed low temperature limit for C. *sinensis*.

Physiological changes occurring during freeze acclimation of citrus include increases in sugar levels, colloid stability, sap concentration, proline, valine, and bound water (Yelenosky, 1985) and decreases in total water, soluble protein, total water content, Ψ_x , freezing point, other amino acids, and reduced glutathione (Young, 1970; Yelenosky, 1978). Drought (Davies et al., 1981) and salinity stress (Syvertsen and Yelenosky, 1988) also increase freeze tolerance.

Weiser (1970) suggested that cold acclimation might be the result of altered gene regulation. Since 1970 changes in gene expression related to freeze acclimation have been reported in several crops including *Pisum sativum* (Weiser et al., 1990), *Bromus inermis* Leyss (Lee et al., 1991), *Arabidopsis thaliana* L. Heynh (Welin et al., 1995) and members of the true citrus group (Cai et al., 1995).

Physiological changes in citrus during freeze acclimation have been thoroughly examined on a long-term (weekly or monthly) basis. Using existing physiological and molecular biology methods, it is now possible to examine early changes in citrus freeze hardiness and gene expression. Our objective was to study changes in freeze hardiness, Ψ_x, and gene expression of *P. trifoliata* during short-term (hours to weeks) exposure to freeze-acclimating temperatures and long-days.

Materials and Methods

Plant material. *Poncirus trifoliata* and *C. jambhiri* seeds were purchased from Willits & Newcomb Inc. (Arvin, Calif.). *C. grandis* seeds were provided by Dr. G.A. Moore. Seeds were germinated under mist in the greenhouse in flats using a 100% perlite growing media and held in the greenhouse for 6 weeks under natural daylight and 24 °C day/ 21 °C nights. Seedlings in flats were then transferred into a Conviron Model E15 growth chamber (Winnipeg, Manitoba, Canada) and grown at 25 °C and 16 h days (PPF=450 μmol m^{-2} s^{-1}) for 2 weeks after which the temperature was decreased to a constant 10 °C.

Assessing freeze hardiness. The procedure used to determine freeze hardiness was essentially the same as in Chapter 3 (page 38 to 39) and is briefly described here with a few modifications. Fully expanded leaves, 2.5 cm stem sections, and 2.5 cm of the root system were harvested from each seedling to be tested using a scalpel. Ten replicates of each tissue sample from separate seedlings were collected at each acclimation (exposure to 10° C) time of 0, 6, 24, 168, and 504 h. Excised seedling tissues were placed in 16X100 mm tubes, and 100 μl of distilled deionized water was added. Tubes were immersed in a precooled (-1 C) Model 2425 glycol bath and circulator (Forma Scientific, Marietta Ohio) and allowed to equilibrate for 30 min. One subsample of all treatments was removed as a nonfrozen control. Water in the bottom of the tubes was then nucleated homogeneously in all tubes by adding a small cloud of

ice crystals. Temperatures in the bath were lowered 0.1 C/min until -6.7 °C was reached. Tubes were held for 1 h at -6.7 °C and then removed. Frozen samples were allowed to thaw overnight at 1 °C in a low temperature incubator to eliminate injury caused by rapid warming. Tubes were removed from the incubator, allowed to reach 20 °C, and initial EL was measured with a model CDM3 conductivity meter (Radiometer, Copenhagen, Denmark). Samples were then autoclaved for 25 min to simulate 100% damage and a second EL measurement was taken. Relative % EL was determined based on the method of Flint et al. (1967) with minor modifications. Relative EL for each sample was determined using the method in Chapter 3 (page 39). Percent electrolyte leakage is positively correlated to the loss of membrane integrity and is generally assumed to be a relative measure of tissue injury. Thus increased relative EL following exposure to -6.7 °C is associated with increased injury due to freezing. This experiment was repeated twice in time using different sets of seedlings.

Ψ_s measurements. Seedlings exposed to the same conditions used for freeze hardiness measurements were removed from the tray and the lower 0.5 cm below the root stem interface was excised using a sterile scalpel and discarded. The stem with leaflets intact was then placed into a pressure chamber (Soil Moisture Equipment Corporation, Santa Barbara, Calif.). Pressure was increased gradually until xylem sap could be seen emerging from the cut end with the use of a 10X hand held lens (Scholander et al., 1965). Ten replicate seedlings were harvested after 0, 6, 24, 168, and 504 h exposure to 10 °C. The

experiment was repeated 3 times on different sets of seedlings. Two sets of seedlings were subsets of those harvested for the EL test and the third set was treated identically, but only used for Ψ_x measurements.

Anthocyanin extraction. Five grams of leaf tissue (harvested after 0, 6, 24, 168, and 504 h exposure to 10 °C) was ground in liquid nitrogen, washed in acetone, centrifuged, and this process was repeated until the acetone was clear after centrifugation. The supernatant was discarded after each wash. The pellet was then resuspended in TNE buffer (100 mM Tris-HCl pH 8.5, 100 mM NaCl, 10 mM EDTA pH 8.5, 0.1% Triton X-100 (v/v), and 15 mM DTT added at use). In some of the samples a blue gray color appeared which could be changed to crimson red by reducing the pH to $\cong$ 5 by adding 5N HCl as modified from Neuhaus et al., (1993). This experiment was repeated twice using a subset of seedlings from the EL experiments.

RNA extraction and analysis. Total RNA was extracted from leaves of *Poncirus trifoliata* seedlings exposed to 10 °C for 0, 24, and 168 h using the method for plants high in phenolic compounds (Schneiderbauer et al., 1991), except that the RNA was precipitated in 1.7 M LiCl instead of being pelleted through a CsCl cushion. Concentration and integrity of the RNA was verified by electrophoresis through an agarose gel and staining with ethidium bromide. Ten μg of total RNA was subjected to electrophoresis in formaldehyde agarose gels (Sambrook et al., 1989) and transferred using a vacuum blotter (Hoefer) to

Hybond N+ membranes (Amersham) with 50 mM NaOH as transfer buffer. Complementary DNA (cDNA) clones for phenylalanine ammonia lyase (PAL) and 4-coumarate: coenzyme A ligase (4CL) (from *Populus deltoides*) were provided by Carl Douglas (Univ. of British Columbia). A cDNA clone for citrus chalcone synthase (CHS) was provided by Diane Luth and Gloria Moore (Univ. of Florida). Clone 24 encoding chlorophyll a/b binding protein (John Davis, unpublished data) served as a positive control for equal loading and hybridization on the blots. The cDNA inserts were amplified by the PCR using forward and reverse sequencing primers, purified using Qiaex resin (Qiagen, Chatsworth, CA), and then labeled with $\alpha^{[32]}$ PdCTP using a random primers DNA labeling kit (Gibco BRL, Gaithersburg, MD). Hybridization and washing were as previously described (Church and Gilbert, 1984) except that the final wash was performed at 55 °C. Northern analysis was performed on RNA extracted from subsets of seedlings from plants in two independent experiments.

Differential display of mRNA. Four μg total RNA extracted from leaves harvested at 0, 6, 24, or 168 h was brought up to 8.5 μl volume with dd water in a thin-walled micocentrifuge tube. Ten μl of the reverse transcriptase master mix (4 μl 5X reverse transcriptase buffer, 2 μl dithiothreitol (DTT) (100 mM), 2 μl d(T)iNN primer (2 μM), 2 μl dNTPs (200 μM)) was added to the tube and heated to 65 °C for 5 min. Tubes were centrifuged briefly and allowed to incubate at 37 °C for 10 min. Reverse transcriptase (1.5 μl MMLV-RT, Gibco-BRL) was added to tubes and reaction mixes were incubated at 37 °C for 1 h. Tubes were then

heated to 85 °C to inactivate the reverse transcriptase. Reactions not used immediately were stored at –20 °C.

Single stranded (ss) complimentary DNA (cDNA) was then used as a template for the PCR. Two μl of ss cDNA was added to 2 μl of a random 10-mer primer (Table 5.1) in a thin walled tube to which 16 μl of the PCR master mix (2 μl 10X PCR buffer, 1.2 μl $MgCl_2$ (25 mM), 2 μl dNTPs (200 μM), 2 μl d(T)iNN primer (same from reverse transcriptase reaction), 1 μl ^{33}P dATP's, and 7.6 μl dd water) was added and PCR carried out at conditions suggested by Liang and Pardee (1992). Following PCR, 4 μl of stop dye (95% formamide, 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol) was added to the tubes. Tubes were heated to 95 °C for 5 min and then quenched on ice. Any PCR reactions not used immediately were stored at –20 °C. Random primers and anchored primer sequences used in mRNA differential display analysis are shown in Table 5.1 and combinations tested and results in Table 5.2.

Reactions were electrophoresed using a Gibco-Brl model SA adjustable sequencing gel system (Gaithersburg, MD) through a 6% acrylamide / 7M Urea gel. Gel temperature was maintained at 60° C throughout the run to enhance band resolution. Gels were dried for at least 2 h and then exposed to autoradiography film for 5 to 8 h.

Table 5.1. Primers used for differential display of mRNA. Primers were purchased from the ICBR core labs at the Univ. of FL, Gainesville.

Primer Name	Sequence 5' to 3'
74[z]	TTT TTT TTT TTT TTT TAT
75[z]	TTT TTT TTT TTT TTT TGA
76[z]	TTT TTT TTT TTT TTT TGG
78[z]	TTT TTT TTT TTT TTT TGT
79[z]	TTT TTT TTT TTT TTT TCA
80[z]	TTT TTT TTT TTT TTT TCG
81[z]	TTT TTT TTT TTT TTT TCC
82[z]	TTT TTT TTT TTT TTT TCT
83[y]	GTT GCG ATC
84[y]	CAA ACG TCG
85[y]	AGG TGA CCG
86[y]	GAC CGC TTG
87[y]	AGC CAG CGA

[z]18mer anchored primer
[y]10mer random primer

Table 5.2. Primer combinations tested for differentially displayed mRNA products and the result. Numbers represent quantity of differentially displayed products using that primer combination, N = not tested.

Anchored Primer No.	Random Primer No.				
	83	84	85	86	87
74	0	0	0	0	0
75	N	0	0	0	N
76	N	0	0	0	N
78	N	0	0	0	0
79	N	0	0	0	0
80	N	0	2	4	0
81	N	0	0	0	N
82	0	0	0	0	0

Results and Discussion

Freeze Hardiness. Injury to *P. trifoliata* leaves and stems was significantly reduced at -6.7 °C following exposure to 10 °C for 6 h (Fig.5.1). Within 6 h of exposure to 10 °C, leaf % EL was reduced by half compared to leaves harvested at time 0 h, a similar decrease was observed in stems. Leaf % EL reached a plateau at 10% after 24 h of acclimation, but then began to increase again after 168 h of acclimation. This increase in % EL coincided with the initiation of leaf drop in the growth chamber. After one week of exposure to 10 °C, stem sections had only 5% EL, eventually showing no relative EL after 504 h when frozen to -6.7 °C. Leaves and stems showed a more rapid decrease in % EL with acclimation than roots. Root tissue showed no significant change in % EL in 6 h, however, there was a one third reduction after 24 h. The degree of freeze acclimation of roots was surprising and is not usually measured because root temperatures rarely fall below 0 °C in commercial citrus growing regions. Following 504 h of 10 °C the remaining leaves and the roots showed 15-20% EL, but % EL was not significantly different between the two.

In order to compare the acclimation of *P. trifoliata* at 10 °C, a very hardy citrus relative, with other less hardy citrus, the freezing injury of *P. trifoliata*, *C. grandis*, and *C. jambhiri* were examined simultaneously (Fig. 5.2). *P. trifoliata* leaves had the least EL before acclimation (0 h) (Fig. 5.2.A). *C. grandis* and *C.*

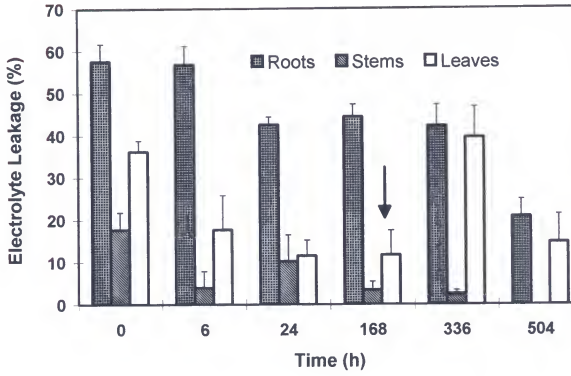

Figure 5.1. Percent electrolyte leakage of roots, stems, and leaves of *P. trifoliata* 'Rubidoux' at -6.7 °C after various freeze acclimation periods at 10 °C. Arrow indicates where leaf drop began to occur. A representative graph is shown. The experiment was repeated twice. Mean electrolyte leakage values ± SE, n=10.

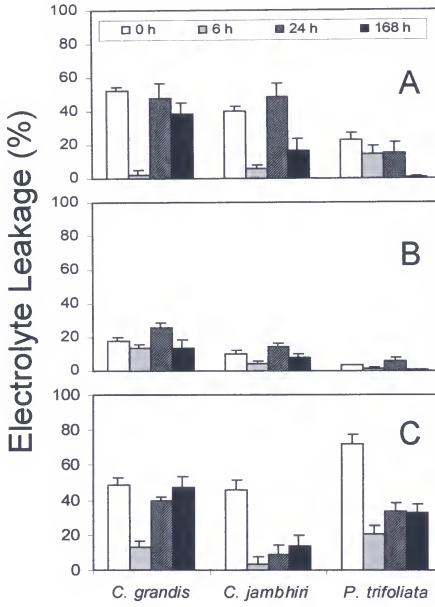

Figure 5.2. Freezing injury of *C. grandis*, *C. jambhiri*, and *P. trifoliata* 'Rubidoux' leaves (A), stems (B), and roots (C) during a one week time course of exposure to long days and 10 °C. Seeds were germinated in the greenhouse and after 3 weeks moved to a growth chamber and allowed to recover at 24 °C days and nights. Temperatures were then dropped to 10 °C continuously. Daylength was kept constant at 16 h. A freeze test was conducted at 0, 6, 24, and 168 h. Tissue samples were frozen to –6.7 °C. Mean electrolyte leakage levels ± SE, n=10.

jambhiri leaves had similar EL, between 40 and 50 % at 0h. All species showed a drop in leaf EL following 6 h exposure to 10 °C which was dramatic for *C. grandis* and *C. jambhiri*. After 24 h of exposure to 10 °C leaf EL levels had returned to levels found in unacclimated leaves with the exception of *P. trifoliata* which had lower EL values. At 168 h *C. grandis* showed a slight significant reduction in leaf EL compared to 0 h, however, *C. jambhiri* showed a 50% reduction as compared to time 0 h. *P. trifoliata* leaves showed almost no EL following 168 h exposure to 10 °C. Electrolyte leakage values for stems were much less than leaf values, but trends were still apparent (Fig. 5.2.B). At 168 h *C. grandis* stems had similar EL values to those at 0 h suggesting that little acclimation had taken place. Both *C. grandis*, *C. jambhiri*, and *P. trifoliata* stems showed an increase in electrolyte leakage values at 24 h. At 168 h, only *P. trifoliata* stems showed a reduction in EL. Roots of all species tested showed a similar drop in EL with the exception of *C. jambhiri,* which showed a substantial drop in EL with exposure to 10 °C.

In summary, acclimation trends for *C. grandis*, *C. jambhiri*, and *P. trifoliata* were what would be expected based on years of field and laboratory freeze observations (see review by Yelenosky, 1985). Both *C. grandis* and *C. jambhiri* acclimated more slowly and to a lesser degree than *P. trifoliata* as determined by using EL as an estimate of freeze hardiness. The only exception came with the unexpected acclimation level observed in *C. jambhiri* roots.

The fact that when all species and tissue types were tested a rapid drop in EL was observed at 6 h suggests that these values might be an artifact of the

technique. Possibly the drop in temperature from 25 °C to 10 °C causes an acute drought stress. It is possible that for a time stomata may be open while roots are unable to take up water due to viscosity changes caused by rapidly chilled seedling flats. However, it could be stresses like this that signal the onset of acclimation mechanisms in citrus and *Poncirus*.

Ψ_x Changes. Ψ_x of the *P. trifoliata* seedlings decreased from -0.75 to -2.0 MPa within 2 h of freeze acclimation with minimum levels occurring at 6 to 8 h (Fig. 5.3). After 24 h at 10 °C, Ψ_x levels remained significantly more negative than at time zero. After one week of exposure to 10 °C, however, Ψ_x levels approached those of nontreated seedlings.

Young (1970), Cooper (1965), and Yelenosky (1975, 1978) observed that exposure to low temperature regimes reduced freeze injury to citrus trees. These observations were based on trees or seedlings that had been exposed to freeze acclimating temperatures for 1 week or more. In this study significant reductions in % EL occurred after as little as 6 h of acclimation. These rapid changes may be due to changes in viscosity of water and membrane changes at low temperatures (Uemura and Yoshida, 1984), but changes in gene expression could also be important. Likewise, changes in Ψ_x have been observed in citrus leaves during acclimation. Ψ_x in 'Valencia' orange leaves on 1-year-old potted plants decreased from -1.8 to -2.0 MPa, but only after 4 weeks of freeze-hardening temperatures (Yelenosky, 1978). Decreases in Ψ_x were also observed

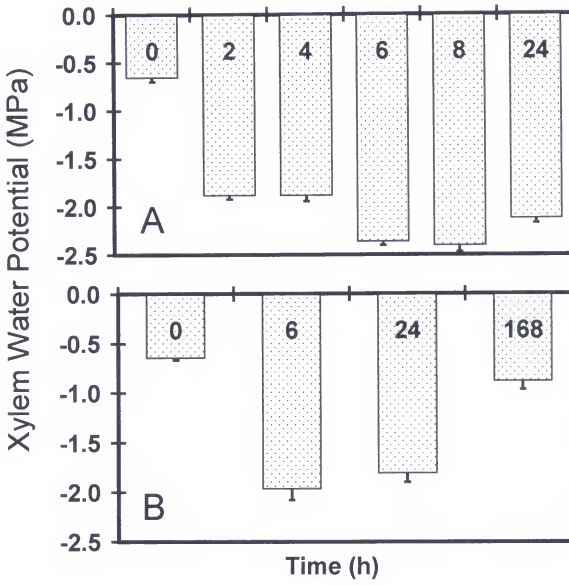

Figure 5.3. Ψ_x of *P. trifoliata* seedlings exposed to 10 °C for various time periods using a pressure chamber. Experiments were repeated three times. Representative graphs of each type of experiment are shown. (A) 24 h time course. (B) 1-week time course. Each data point represents the mean of 10 seedlings ± SE.

in 'Carrizo' citrange leaves after 6 weeks of successively lower temperature exposure to the roots (lowest temp was 5 °C) (Wilcox et al., 1983). *P. trifoliata* (the male parent of 'Carrizo' citrange) seedlings showed a similar magnitude of change in Ψ_x after only 6 h of acclimation (Fig. 5.3). Nevertheless, Ψ_x values approached those of nontreated seedlings after 1 week of acclimation. In previous studies Ψ_x remained low throughout the acclimation period (Yelenosky, 1978; Wilcox et al., 1983). The increase in Ψ_x may have occurred due to stomatal closure at low temperatures, which stabilized Ψ_x.

Pigment changes. During exposure to 10 °C, a red pigment became visible first at the junction of the petiole and leaflets of *Poncirus trifoliata* seedlings (6 h) and later on the leaves (168 h) and in the stem (504 h) (data not shown). The pigment was extractable in an aqueous solution and showed a pH-dependent color change from blue (high pH) to pink (low pH) and was therefore hypothesized to be an anthocyanin based on previous studies (Neuhaus et al., 1993). Anthocyanin-like compounds were detectable in leaf extracts after 168 h of exposure to 10 °C and in stem extracts after 504 h (data not shown).

RNA changes. In other studies where flavonoid biosynthesis was induced by environmental stresses, messenger RNA levels of genes in the phenylpropanoid pathway (PAL, CHS, 4CL) increased before or simultaneous with pigment accumulation (Christie et al., 1994; Leyva et al., 1995). Christie et al., (1994) showed increased levels of PAL, 4CL, and CHS mRNA in 24 h when

Zea mays seedlings were exposed to 10 °C and increased anthocyanin levels in 10 °C treated seedlings as compared to controls at 7 days. Leyva et al. (1995), demonstrated that PAL and CHS transcript levels increased in *Arabidopsis thaliana* when exposed to 4 °C. To determine if this was also occurring in citrus, cDNAs encoding enzymes in the flavonoid biosynthesis pathway were used to probe RNA extracted from *P. trifoliata* seedlings at 0, 24, and 168 h. Hybridization was observed to all three of the probes tested (PAL, CHS, 4CL) as well as to the positive control probe (CAB). However, no increases in steady state RNA levels were observed relative to the 0 h treatment (Fig. 5.4.). This result suggests that processes other than PAL, CHS, and 4CL induction are involved in citrus pigment accumulation, or that transient increases in mRNA levels occurred that were not detected at 24 and 168 h.

Anthocyanin production often occurs concomitantly with freeze acclimation (Parker, 1963). *Cornus stolonifera* L. (Van Huystee et al., 1967), *Cicer arietinum* L. (Singh et al., 1995), and *Pinus contorta* Dougl. var. latifolia Engelm. (Camm et al., 1993) showed increases in anthocyanin production in leaves and/or bark concomitant with increases in freeze hardiness. Anthocyanin accumulation in *Hedera helix* (Steponkus and Lanphear, 1969) and chickpea (Singh et al., 1995) was incidental to increases in freeze hardiness. Parker (1963) suggested that increases in anthocyanin may not be a direct response to acclimating temperatures, but may be an indirect response to increased sugar levels. Whatever the primary cause of anthocyanin accumulation, it is unlikely that these pigments promote freezing acclimation in citrus. However, it is clear that early

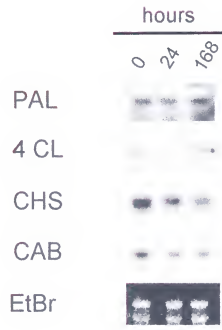

Figure 5.4. Northern blot analysis of total RNA (10 μg/lane) extracted from *P. trifoliata* 'Rubidoux' seedling leaves during a one week exposure to 10 °C. Ethidium bromide (EtBr) was used to evaluate RNA quantity and quality among lanes. Hybridization probes included: 4-coumarate:coenzyme A ligase (4CL), chalcone synthase (CHS), chlorophyll a/b binding protein (CAB), and phenylalanine ammonia lyase (PAL). Blots were washed at moderate stringency. Northern blots were completed on RNA extracted on two separate occasions from two separate experiments with similar results.

biochemical and metabolic changes were taking place in association with acclimation temperatures.

Differential display of mRNA was used to examine if any of the early changes we observed based on EL and Ψ_x were accompanied by early changes in the relative abundance of different mRNA species. There are putative up and down regulated mRNA transcripts at both 6 (data not shown) and 24 h (Fig. 5.5), giving support to the hypothesis that changes in gene regulation may be occurring very early in *P. trifoliata* seedlings exposed to 10 °C. However, differentially displayed transcripts were rare suggesting that potentially only a few transcripts are changing in abundance with exposure to acclimating temperatures.

Percent EL, Ψ_x, and some mRNA abundance levels change rapidly, within hours, when *P. trifoliata* seedlings are exposed to 10 °C and long days. In addition, there are gross pigment changes that are detectable in crude extracts after 1 week. In an effort to understand citrus freeze acclimation, and thus possible mechanisms of freeze acclimation in other subtropical crops, short term changes that occur in hours and even minutes should be examined. Biochemical changes during freeze acclimation may be initiated long before they are manifested at the cell or whole-organ level.

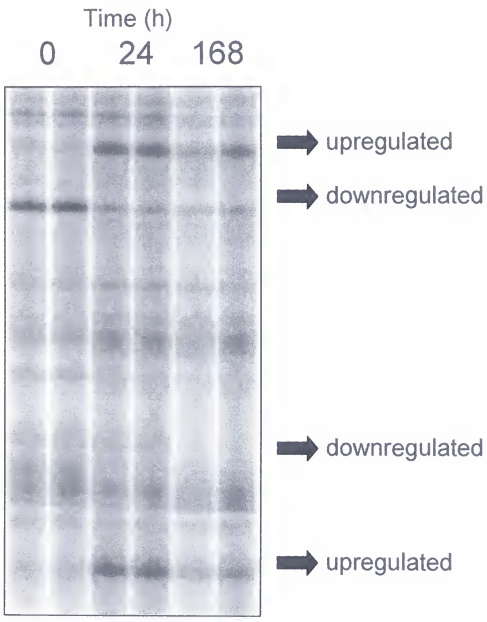

Figure 5.5. Autoradiogram of putative differentially displayed *P. trifoliata* 'Rubidoux' mRNA exposed to 10 °C and 16 h days for 0, 24, and 168 h. Messenger RNA was extracted *P. trifoliata* leaves and reverse transcribed with MMLV and an anchored primer No. 80. Complimentary DNA was then amplified using PCR using the same anchored primer and random primer No. 86. Duplicate samples were electrophoresed in a 6% polyacrylamide denaturing urea gel. The gel was then dried and exposed to X-ray film for 8 h.

CHAPTER VI
CONCLUSIONS

The study of citrus freeze hardiness is important since periodic freezes result in substantial economic loss in several important citrus growing regions. Florida, in particular, was severely affected by several advective freezes in the 1980s which resulted in over 1 billion dollars worth of damage.

The effects of irrigation scheduling and nutrient application frequency on the freeze hardiness and acclimation of young 'Hamlin' orange trees were examined. Irrigation had a significant effect on freeze hardiness during the winter of 1994. Irrigation at 20% soil water depletion (SWD) through growth flush 1 and 45% SWD thereafter made trees significantly more hardy than those that received more water during the year. In addition, this treatment significantly delayed the second growth flush of 'Hamlin' trees in 1994. Fertilizer application frequency had a significant effect on plant height by the end of summer 1995, greater amounts of N applied Apr to Sept produced taller trees than the same amount of N applied in less amounts over a greater time period. These data suggest that altering irrigation scheduling programs could result in improved freeze hardiness of young citrus trees during periods of low rainfall.

After 4 years of field evaluations, the following conclusions can be made about the freeze hardiness, acclimation, and growth characteristics of USDA intergeneric citrus scion breeding lines 'US 119' and selection 17-11. 'US 119' and 17-11 were both hardier than 'Hamlin' orange as determined by leaf disc EL. Both showed freeze hardiness similar to that of satsuma mandarin. Interestingly, 17-11 was significantly hardier than satsuma or 'US 119' during several time periods during the 4-year study. Growth characteristics of 'US 119' and selection 17-11 were similar.

Finally, some early physiological and molecular changes of *P. trifoliata* seedlings exposed to low acclimating temperatures were examined. Decreases in EL were seen with as little as 6 h exposure to 10 °C and EL continued to decrease for up to 168 h. Xylem water potential initially decreased from –0.6 to –2.0 MPa after 6 h, but returned to initial levels following 168 h exposure to 10 °C. In addition, several putative differentially displayed mRNA products were seen as soon as 6 h following exposure to acclimating temperatures, suggesting that at least some alterations in gene regulation or mRNA turnover may be occurring very early in the acclimation process. These data suggest that future basic research on citrus freeze hardiness should focus on very early as well as later time intervals following exposure to acclimating temperatures.

In summary, this research suggests that there are potential opportunities to improve citrus freeze hardiness from the most applied cultural practices and traditional breeding programs to more basic studies.

APPENDIX A
TEMPERATURE AND RAINFALL DATA FOR GAINESVILLE, FL FROM FALL 1993 TO SPRING 1997

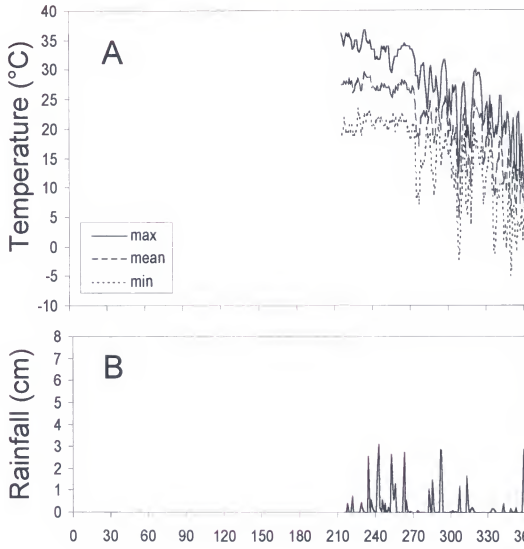

Figure A.1. Temperature (A) and rainfall data (B) for Gainesville, FL for fall of 1993. Values obtained from the Weather Office at the Agronomy Department, IFAS, University of Florida, Gainesville, FL 32611.

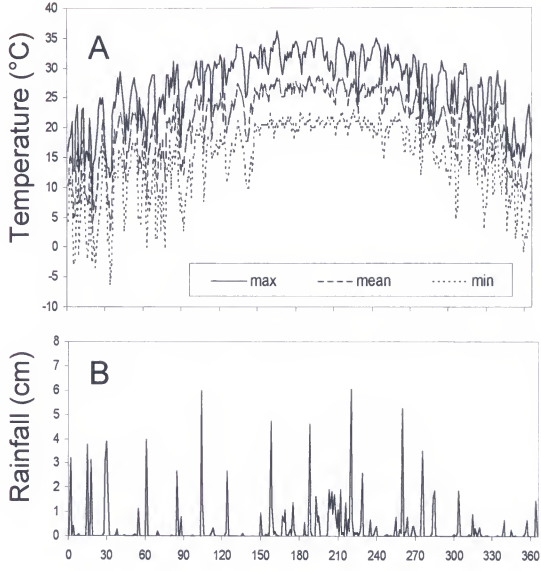

Figure A.2. Temperature (A) and rainfall data (B) for Gainesville, FL for 1994. Values obtained from the Weather Office at the Agronomy Department, IFAS, University of Florida, Gainesville, FL 32611.

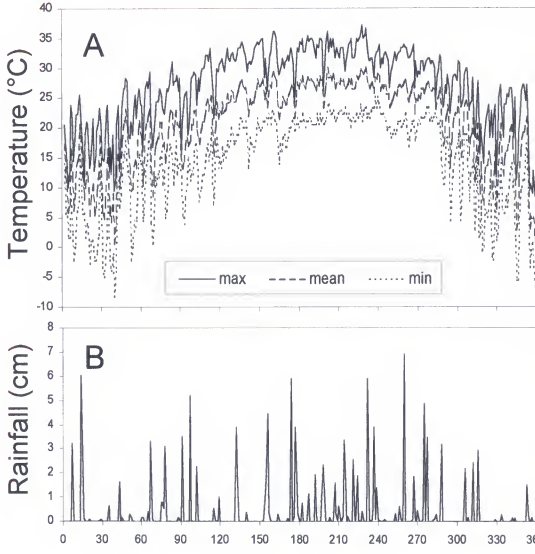

Figure A.3. Temperature (A) and rainfall data (B) for Gainesville, FL for 1995. Values obtained from the Weather Office at the Agronomy Department, IFAS, University of Florida, Gainesville, FL 32611.

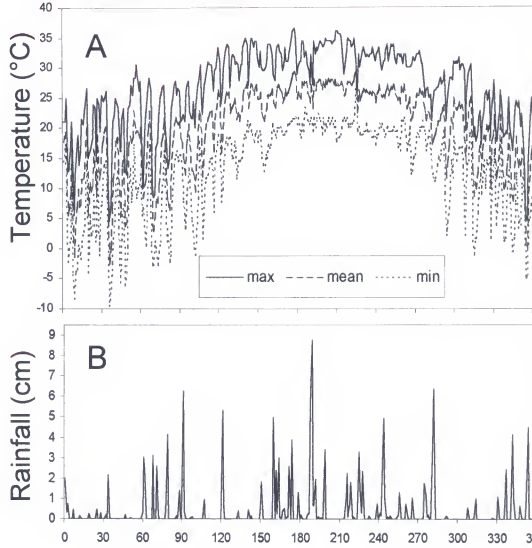

Figure A.4. Temperature (A) and rainfall data (B) for Gainesville, FL for 1996. Values obtained from the Weather Office at the Agronomy Department, IFAS, University of Florida, Gainesville, FL 32611.

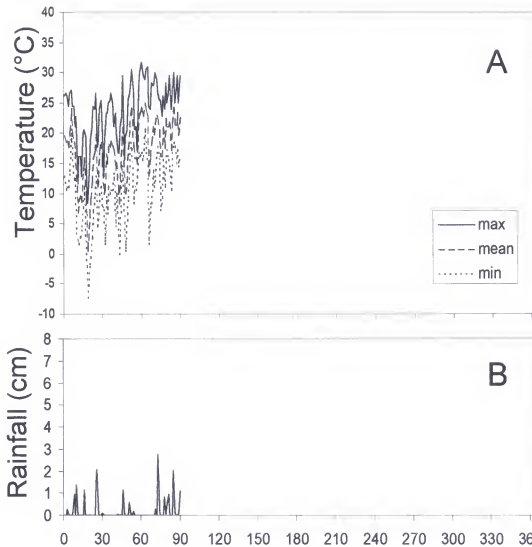

Figure A.5. Temperature (A) and rainfall data (B) for Gainesville, FL for spring of 1997. Values obtained from the Weather Office at the Agronomy Department, IFAS, University of Florida, Gainesville, FL 32611.

APPENDIX B
COMPARISON OF THE ELECTROLYTE LEAKAGE TECHNIQUE WITH WHOLE TREE FREEZING

Introduction

In order to study freeze acclimation, deacclimation and hardiness it necessary to have a reliable measure of freeze injury. Electrolyte leakage (EL) measurements have often been a key component in determining membrane damage following freezes (Dexter et al., 1932; Flint et al., 1967; Maier et al., 1994). In the study of citrus freeze hardiness leaf EL data have been analyzed to yield leaf killing points (LKPs) to predict freeze hardiness in the field (Wiltbank and Oswalt, 1983; Maurer and Davies, 1994). In this study leaf disc EL data was used directly to determine freeze hardiness.

In order to determine if the leaf disc EL method had the necessary resolution to significantly separate differences in citrus and related genera, a series of freeze tests using *Poncirus trifoliata* and *C. grandis* seedlings were conducted. *P. trifoliata* is a very hardy deciduous relative of citrus and *C. grandis* is one of the more freeze sensitive members of the citrus genus. Results using the leaf disc EL method were also compared to whole plant freeze tests on 'Hamlin' orange, 'Cleopatra' mandarin, and 'Swingle' citrumelo.

Materials and Methods

Experiment 1. *C. grandis* and *P. trifoliata* trees for this experiment were kindly provided by Dr. G.A. Moore and were grown and maintained by Ilhami Tozulu. Plants of both species were grown in citri-tubes, were of similar size, and approximately 4-months old. Trees were either acclimated for 2 weeks

under 21 °C days / 10 °C nights, a typical artificial hardening regime for citrus (Yelenosky, 1985), or grown at 25 °C days and nights in an identical growth chamber. Both sets of young trees were subject to 12-h days and were watered as needed. Leaves were harvested at the end of the 2-week period and a freeze test was conducted in an identical manner to freeze tests discussed in Chapter 3 (page 38 to 39). The experiment was repeated twice.

Experiment 2. Two sets greenhouse grown 2-year-old 'Hamlin' orange on 'Swingle' citrumelo, 'Cleopatra' mandarin, and 'Swingle' citrumelo were either acclimated for 6 weeks under 21 °C days / 10 °C nights or left in the greenhouse at continuous 26 °C and ambient light conditions. Ten leaves from 'Hamlin', 'Swingle,' and 'Cleopatra' trees were harvested and used to determine EL as in Chapter 3 (page 38 to 39) at –3, -6, and –9 °C. In addition, 5 trees of each species, both acclimated and non-acclimated, were then placed into coolers and vermiculite used to provide insulation in, around, and 5 cm above the soil line in the pots. Coolers were then placed in a walk-in freeze chamber. Temperature was lowered from 25 to –6.0°C at 5 °C/ h and held at –6 °C for 1 h. Temperature was then raised 1 °C/ h to 21 °C following which all trees were returned to the greenhouse where leaf drop and stem dieback were determined. The minimum root temperature during the freeze test as monitored by 3 thermocouples was 9.76 °C. Ten days following the freeze test % leaf drop and % stem dieback was recorded for all trees.

Results and Discussion

Experiment 1. Acclimated *P. trifoliata* leaves showed significantly less EL (were more freeze hardy) at –4, -6, and –8 ° C than non-acclimated *P. trifoliata*, non-acclimated *C. grandis*, and acclimated *C. grandis* (Fig. B.1). Acclimated *C. grandis* had less EL than its non-acclimated counterpart at –4 °C, but there no decrease at –6° and only a slight decrease at –8°C. This experiment indicated that this EL technique could detect differences in freeze hardiness between acclimated and non-acclimated trees of both freeze hardy and freeze-resistant citrus and near relatives. In addition, during the second repetition of this experiment the technique was sensitive enough to detect a loss in freeze hardiness that was caused by a malfunctioning growth chamber (data not shown).

Experiment 2. There were no significant differences in EL at –4° C among trees tested (Fig. B.2.A). The greatest separation in % EL occurred at –6° C (Fig. B.2.B). 'Swingle' had a mean of 8% EL, while 'Cleopatra' had a mean of 26% EL. 'Hamlin' which had a mean of 46% EL was the least hardy by this technique. There was no significant difference in EL % between 'Hamlin' and 'Cleopatra' at –9°C, however, 'Swingle' had a reduction in EL by 50% (Fig. B.2.C). Based on the leaf disc EL method 'Swingle' citrumelo was the most

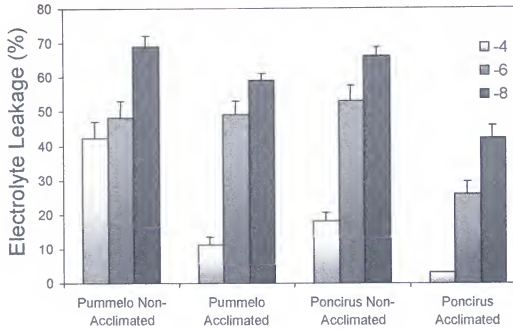

Figure. B.1. Electrolyte leakage of acclimated and non-acclimated *Poncirus trifoliata* and *C. grandis* (pummelo) leaves at -4, -6, and -8 °C. Acclimated plants were grown under 21 °C days/ 10 °C nights and 12 h daylength. Non-acclimated plants were kept in an identical growth chamber at 26 °C and 12-h daylength. Bars represent SE, n=10.

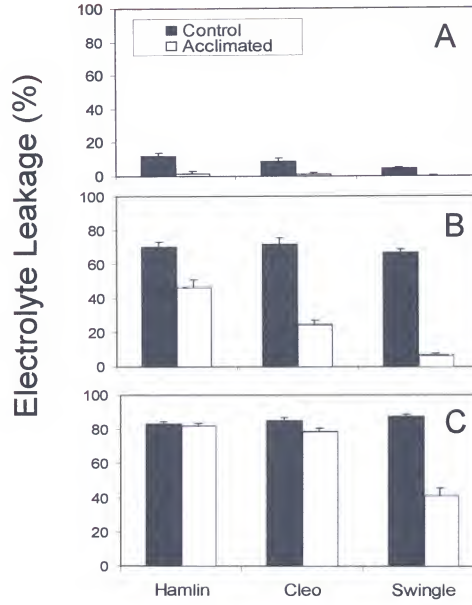

Figure. B.2. Electrolyte leakage of acclimated and non-acclimated 'Hamlin' orange, 'Cleopatra' mandarin, and 'Swingle citrumelo leaves. Results based on leaf discs frozen to -3 (A), -6 (B), and -9 °C (C). Acclimated plants were grown under 21 °C days/ 10 °C nights and 12 h daylength. Non-acclimated plants were kept in an identical growth chamber at 26 °C and 12 h daylength. Bars represent SE, n=10.

hardy tree tested, followed by 'Cleopatra' mandarin and 'Hamlin' orange which was the least hardy (Fig. B.2).

All non-acclimated intact trees frozen to –6 °C showed 0 or near 0 % leaf drop, but the leaves were discolored, dehydrated, and dead (Fig. B.3). Acclimated 'Hamlin' orange leaves showed 80% leaf drop, but as expected they abscised at the petiole leaf junction a sign of increased freeze hardiness in citrus (Frederick Davies, personal communication). Acclimated 'Cleopatra' and 'Swingle' leaves showed 42 and 0% leaf drop, respectively suggesting that acclimated 'Swingle' tree leaves are fully hardy down to –6° C. Non-acclimated stem dieback ranged from 58 to 74% for all tree types, but among acclimated trees only 'Cleopatra' mandarin stems showed any damage (8%). This was likely because 'Cleopatra' mandarin was still actively growing during the acclimation period. All trees began a growth flush approximately 8 – 11 days following the freeze test.

These results provide a comparison of EL data to regrowth tests for 'Hamlin' orange, 'Cleopatra' mandarin, and 'Swingle' citrumelo. Damage values observed for 'Hamlin' at –6 ° are similar to those reported by Yelenosky (1990), although he reported no leaf drop. Both methods show differences in hardiness among the species that are in general agreement with those previously reported (Yelenosky, 1985).

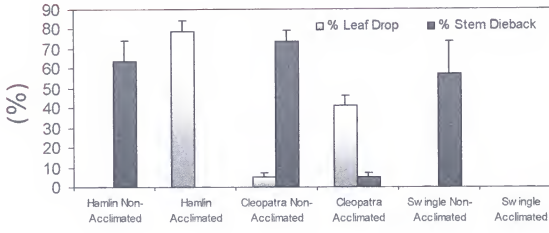

Figure B.3. Leaf drop and stem dieback of non-acclimated and acclimated 'Hamlin' orange, 'Cleopatra' mandarin, and 'Swingle' citrumelo 10 days after a whole plant freeze test to -6 °C for 1 h. Bars represent SE, n=5.

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BIOGRAPHICAL SKETCH

Milton E. Tignor, Jr. was born to Milton and Shirley Tignor on April 2, 1968, in Richmond, Virginia. His father almost immediately gave him the nickname "Buddy." He was raised by his parents on his maternal grandfather's tobacco farm in Chesterfield County, Virginia, attended Manchester High School and graduated under the optional science curriculum in June 1986. He earned a Bachelor of Science in horticulture in May 1990 and a Master of Science in horticulture specializing in cold acclimation in September of 1992, both from Virginia Polytechnic Institute and State University, Blacksburg, Virginia. Beginning in 1992 he studied for one year at Oregon State University (Corvallis) taking courses in molecular biology, environmental physiology, and plant breeding. He arrived at the University of Florida (Gainesville) in August of 1993 and entered his doctoral studies in the Horticultural Sciences Department.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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August 1997

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